The Effect of Individual Amino Acid Intakes on Clinical Infection Risk in Very Preterm Neonates and How Modifying Amino Acid Formulation Impacts Early Postnatal Metabolic Adaptation in This Population

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Master of Philosophy by Keziah Rose Davies

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Abstract

Background

Very preterm neonates (VPN) depend on parenteral nutrition (PN) to support postnatal growth. Evidence from critically ill, term neonates indicates that the immediate provision of parenteral amino acids (AA) may be harmful and increase the risk of infection. Inhibition of autophagy, a catabolic process important to innate immunity, has been proposed as a mechanism. This thesis aims to overview the importance of parenteral AA and understand how modifying AA formulation might impact early postnatal metabolic adaptation, exploring different methodologies. The chapters of this thesis investigate whether essential AA (EAA) overprovision is associated with infection using data generated from local research. This is complemented with a systematic review focusing on leucine, an EAA known to inhibit autophagy through the mTOR pathway.

Methods

First, we conducted a secondary analysis of data from 150 infants recruited for the SCAMP nutrition study. We compared the total parenteral AA intake and individual plasma AA levels between infants who developed late-onset sepsis (LOS) and those who did not. Secondly, a systematic review was performed to quantify the relationship between parenteral leucine intake and plasma leucine levels in VPN. Lastly, we explored the feasibility of transcriptomics to examine postnatal innate immune development. We conducted a preliminary transcriptomic analysis using data from the PAINT study, focusing on changes in the expression of mTOR-related transcripts.

Results

Further analysis of the SCAMP data showed no significant differences in total parenteral AA intake or plasma EAA levels between infants who developed LOS and/or necrotising enterocolitis (NEC) and infants who did not. There was no evidence of greater leucine overprovision in infants who developed LOS. Reduced plasma arginine and glutamine may explain why some infants are predisposed to infection.

The systematic review included 12 articles, which collectively studied 650 VPN. The dose-concentration relationships of leucine content (%) and absolute leucine intake (mg/kg/d) with plasma leucine level (µmol/L) showed significant, moderately positive correlations. Regression analysis indicated that absolute leucine intake was the stronger predictor of plasma level ($p < 0.05$). The review provided equations that could be used to calculate the appropriate (reduced) parenteral leucine intake for the desired reference range.

Finally, the preliminary transcriptomic analysis showed minimal, isolated changes in mTOR-related gene expression between day 3 and day 10 of life. Investigating neonatal PN with reduced leucine and other EAA appears unlikely to have substantial clinical effects on the innate immune system following changes in mTOR gene expression.

Conclusion

Overall, the evidence presented suggests leucine overprovision does not confer an increased infection risk in VPN caused by deficient autophagy. However, the overall thesis has emphasised the potential clinical importance of designing and testing an optimised parenteral AA solution to support postnatal immune development. Future multi-omics research is needed to understand the changes (metabolic, inflammatory and immune) underpinning early postnatal adaptation concerning PN.

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Contribution to the Thesis

The main bulk of this thesis was done by the candidate, Keziah Davies. The following people have also made an important contribution to the work stated in this thesis:

Chapter 2: SCAMP data analysis

Role	Contributor
Principal Investigator	KD
Data collection	KD
Statistical analysis plan	KD, CM
Data analysis	KD
Supervision	CM and MT

Chapter 3: Systematic Review

Role	Contributor
Principal Investigator	KD
Methodology design	KD, CMP, CM
Literature search	KD
Article screening and selection	KD
Development of data extraction forms	KD and CM
Data extraction	KD
Quality assessment	KD
Data analysis	KD
Supervision	CM and MT

Chapter 4: Transcriptomic Analysis

Role	Contributor
Principal Investigator	KD
Transcriptomic analysis plan	KD and EC
Data analysis	EC
Supervision	CM and MT

CM – Professor Colin Morgan

CMP – Dr Chandini Menon Premakumar

EC – Dr Eva Caamano-Gutierrez

KD – Keziah Davies

MT – Professor Mark Turner

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List of Abbreviations

Abbreviation	Meaning
AA	Amino acid(s)
AIO	All-in-one
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Atg	Autophagy-related genes
BC	Blood culture
BCAA	Branched-chain amino acid
BPD	Bronchopulmonary dysplasia
BUN	Blood urea nitrogen
CASP	Critical Appraisal Skills Programme
CEAA	Conditionally essential amino acid
CGA	Corrected gestational age
CI	Confidence interval
CLABSI	Central line-associated bloodstream infection
CLD	Chronic lung disease
CM	Colin Morgan
CNS	Central nervous system
CRF	Case report form
CS	Caesarean section
CVC	Central venous catheter
Cys	Cysteine
D3	Day 3
D10	Day 10
DBS	Dried blood spot
DC	Dendritic cell
EAA	Essential amino acid
EAPS	European Academy of Paediatric Societies
EBM	Expressed breast milk

ELBW	Extremely low birth weight
EOS	Early-onset sepsis
EPaNIC study	Early Parenteral Nutrition Completing Enteral Nutrition in Adult Critically Ill Patients
ESPGHAN	European Society for Paediatric Gastroenterology Hepatology and Nutrition
ETT	Endotracheal tube
FcRn	Neonatal Fc receptor
FDR	False detection rate
GBD	Global Burden of Disease
GBS	Group B Streptococcus
GI	Gastrointestinal
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GRADE	Grading of Recommendations, Assessment, Development and Evaluations
HC	Head circumference
His	Histidine
HPLC	High-performance liquid chromatography
ICU	Intensive care unit
IEC	Ion-exchange chromatography
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
Ile	Isoleucine
IOL	Induction of labour
IPA	Ingenuity Pathway Analysis
IQR	Interquartile range
IV	Intravenous
KD	Keziah Davies
Leu	Leucine
LOS	Late-onset Sepsis
LWH	Liverpool Women's Hospital
Lys	Lysine
MeSH	Medical Subject Headings
Met	Methionine

mTOR	Mammalian Target of Rapamycin
mTORC1	Mammalian Target of Rapamycin complex 1
mTORC2	Mammalian Target of Rapamycin complex 2
N	Nitrogen
NCEPOD	National Confidential Enquiry into Patient Outcome and Death
NDO	Neurodevelopmental outcome
NEAA	Non-essential amino acid
NEC	Necrotising enterocolitis
NG	Nasogastric
NICU	Neonatal intensive care unit
NK	Natural Killer cell
NO	Nitrous oxide
OFC	Occipitofrontal circumference
P-PROM	Preterm Premature Rupture of Membranes
PAINT	Preterm Arginine INTake
PAINT18	Preterm Arginine INTake 18
PEPaNIC study	Early versus Late Parenteral Nutrition in the Pediatric Intensive Care Unit
Phe	Phenylalanine
PICC	Peripherally inserted central catheter
PICO	Population, Intervention, Comparison, Outcome
PICU	Paediatric intensive care unit
PKU	Phenylketonuria
PN	Parenteral Nutrition
PNALD	Parenteral nutrition associated liver disease
PPHN	Persistent pulmonary hypertension of the newborn
PRISMA-P	Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols
Pro	Proline
QAT	Quality assessment tool
QIP	Quality improvement project
RCPCH	Royal College of Paediatrics and Child Health
RCT	Randomised control trial
RoB	Risk of bias
ROP	Retinopathy of prematurity
SAM	Significance analysis of microarrays

SCAMP	Standardised, Concentrated, Added Macronutrients Parenteral Nutrition
scNPN	Standardised, Concentrated neonatal PN
SD	Standard Deviation
SEM	Standard Error of the Mean
Ser	Serine
SGA	Small for gestational age
SmPC	Summary of product characteristics
TF	Trophic feeding
Thr	Threonine
TPN	Total Parenteral Nutrition
Trp	Tryptophan
TT1	Tyrosinemia type 1
Tyr	Tyrosine
UoL	University of Liverpool
UVC	Umbilical vein catheter
Val	Valine
VLBW	Very low birth weight
VPN	Very preterm neonate(s)
WHO	World Health Organisation

Chapter 1: Introduction

1.1. Parenteral Nutrition

The term parenteral nutrition (PN) refers to the intravenous (IV) administration of amino acids (AA), glucose, lipid emulsion and micronutrients (1). Total parenteral nutrition (TPN) is defined as nutrition delivered solely via the IV route. PN is indicated in patients who cannot tolerate enteral feeds or in cases when enteral nutrition provides insufficient total protein and energy to meet requirements. This thesis will discuss the use of PN in the neonatal population in further detail. PN should be considered for most preterm neonates, particularly those born with a very low birth weight (VLBW, <1500g), as enteral feeds are often minimal and poorly tolerated (2). In addition to prematurity, PN is also used during periods of acute gastrointestinal malfunction (e.g. post-surgical patients or patients with intractable diarrhoea), which preclude enteral nutrition. PN is a short-term bridge to provide nutritional support until complete enteral nutrition can be provided.

PN should be considered a pharmaceutical product, and the highest standards must be applied to prescription and administration. PN solutions contain around 50 or more individual components meaning they are amongst the most complex pharmaceutical preparations (3). The solutions can be produced in an aseptic, in-house hospital manufacturing unit or purchased from pharmaceutical companies that industrially produce ready-made formulations. The process of providing PN is expensive, technically demanding and fraught with potential complications. Safe execution of PN administration requires a multidisciplinary team including doctors, nursing staff, dieticians, pharmacists, and other pharmacy team members (4). When deciding to commence PN, clinicians must consider the balance of benefits versus risks for all patients.

1.2. Preterm Infants

The World Health Organisation (WHO) defines preterm birth as the birth of an infant before 37 completed weeks of gestation. The incidence of preterm birth is increasing; between 2010 and 2019, the percentage of live births classified as preterm in England and Wales rose from

7.1 to 7.8% (5). Bliss, a charity for babies born premature or sick, equates this to approximately 60,000 cases of preterm birth in the UK each year (6). Very preterm neonates (VPN) are a subgroup of premature infants born between 28 and 32 weeks' gestation. Interestingly, the percentage of infants classed as very preterm remained consistent across the decade at 0.8% (5). However, very preterm birth is often a result of a complicated pregnancy, and these infants may have health issues before birth (7).

Preterm birth has various potential aetiologies, which fall into three main categories: Preterm birth following spontaneous preterm labour, preterm birth following preterm premature rupture of membranes (P-PROM) and clinician-initiated preterm delivery (8). Spontaneous preterm labour is considered a syndrome initiated by multiple mechanisms and changes in the uterus, including infection, cervical disease, decidual haemorrhage and uterine overdistension in the case of multiple pregnancies (9). A previous preterm birth is a significant risk factor for subsequent preterm deliveries. P-PROM complicates 1% of deliveries and is associated with 30-40% of preterm births. There are risks associated with placental abruption and chorioamnionitis (10). Lastly, a premature infant can be delivered by medical intervention if obstetrical complications jeopardise the health of the fetus or mother. Improved and more intensive obstetric management and developments in neonatal care have resulted in a movement towards earlier induction of labour (IOL) and delivery by caesarean section (CS).

Thanks to progress in neonatal intensive care practices, more than 90% of infants born at 29 weeks' gestation survive the neonatal period, defined as the first 28 days of life (11). However, for extremely preterm infants born at 25 weeks, only 66% survive to discharge, falling further to 40% survival at 24 weeks' gestation (12). Prematurity remains the leading cause of perinatal mortality and morbidity (13), and gestational age is the strongest predictor of adverse neonatal outcomes (14). Although most preterm babies born >28 weeks survive, they face an increased risk of long-term neurodevelopmental impairment (15), respiratory morbidity (16) and gastrointestinal complications (17). The rise in the preterm birth rate is a considerable concern, and there is a clear clinical need to focus research on combating complications of prematurity.

1.3. Role of neonatal PN

The birth of a preterm infant should be considered a nutritional emergency (18). Malnutrition amongst VPN does not appear immediately life-threatening compared the acute cardio-respiratory neonatal emergencies which present shortly after very preterm birth and require immediate resuscitation. Still, evidence suggests that even a single day of starvation can be detrimental to the long-term development of a preterm infant (19). VPN have limited glycogen and fat stores at birth coupled with high energy and protein requirements to sustain growth. Additionally, notably higher rates of energy expenditure are reported in neonates compared with adults (50 kcal/kg/d versus 25 kcal/kg/d), partly attributable to heat loss and a high surface area to volume ratio (20). Therefore, the primary aim of neonatal PN is to supply nutrient intakes that can support extrauterine growth at a rate matching the rapid acceleration in fetal weight gain (17-20 g/kg per day) occurring in-utero during the third trimester (21). Fetal weight increases three-fold during this period.

Virtually all preterm infants <29 weeks' gestation or <1200g require an interim period of PN, the length of PN dependency depends on gestational birth weight and comorbidity. The mean period of neonatal PN (>75% all nutrition) in these (surviving) infants is 15.6 days (22,23). In the 2006 EPICure2 national cohort study of infants born between 22 and 26 weeks' gestation (n = 3378), all but one of the surviving babies received PN in the days after birth (12). The data indicates that most preterm infants remain PN-dependent for the longest and constitute the bulk of PN or intensive care days in the NICU, though the surviving patient numbers are small. Even within the preterm population, Van den Akker et al. demonstrated that early AA administration had greater beneficial effects on premature boys than girls (adjusted odds ratio = 6.2, 95% confidence interval (CI) = 1.0-38.0) (24). Optimal nutrition of VPN should meet these infants' unique and rapidly changing requirements, supporting early postnatal growth.

Achieving this objective is often challenging due to the consequences of preterm birth. VPN are more likely to be already growth restricted at birth (25), which may well represent a successful adaption of the fetus to nutrient insufficiency. Readaptation to higher nutrient intakes may be required before postnatal growth begins, a theory supported by animal studies (26,27). Postnatally, pathological events (e.g. hypothermia, hypotension, acidosis, infection

and surgical intervention) and pharmacologic interventions such as caffeine (28) and corticosteroids (29) can all limit the impact of PN and impede growth. Specific neonatal morbidities (ventilated and oxygen-dependent infants) may increase metabolic demand by as much as 25% compared with controls (30–32).

Consequently, postnatal growth failure remains a common and urgent problem for prematurely born neonates. Although multifactorial, growth failure can be partially explained by the iatrogenic nutritional deficit that develops after premature birth. The deficit results from a difference between the energy and protein intake required to mimic fetal growth rates and the actual nutrient intakes delivered to preterm infants (33). In the second and early third trimester, the estimated placental nutrient transfer is 8-10 mg/kg/min of glucose and 3.6 to 4.8 g/kg/d of AA. However, VPN receive significantly less than this postnatally (27). Nutrient deficits are most remarkable in the first week of life but continue accumulating through the first month. The deficit is compounded by delays in the initiation of PN, as in the absence of sufficient nutrition, an infant will begin to catabolise protein to meet basic energy demands. An infant receiving only IV glucose catabolises approximately 1-2% of bodily protein sources each day (34). Essentially, a catabolic state creates a widening gap in lean body mass between a neonate and a fetus of equivalent gestation.

1.3.1. A note on enteral nutrition in preterm infants

Human breast milk is widely accepted as an unequalled form of nutrition for neonates, full-term and preterm (35). It provides well-balanced macronutrients for growth and vital bioactive factors for immune maturation and healthy microbial colonisation (36,37). Unlike PN and infant formula, which are standardised with a fixed and narrow composition, human breast milk composition is dynamic. Composition varies diurnally, over lactation, and between mothers and populations (38). Colostrum, the milk produced in the first few days postpartum, is rich in immunologic factors, including secretory immunoglobulin A (IgA), leucocytes and lactoferrin (39). Human lactoferrin, an iron-binding protein, is being investigated as a novel therapeutic agent because of its antioxidant and antimicrobial properties (40).

Preterm babies benefit from human breast milk nutritionally, immunologically and developmentally. The protein content of breast milk obtained from mothers who deliver preterm is significantly higher than that of mothers who deliver at term (37). Nevertheless, human breast milk does not provide sufficient nutrition for the very low birth weight (VLBW) infant when fed at the usual feeding volumes. Consequently, breast milk is often fortified with additional nutrients, particularly protein, calcium, and phosphate, to meet the high requirements (41). However, VPN experience a period of transient gut immaturity and have an undeveloped suck-swallow reflex, a vital feeding mechanism that develops fully by 34 weeks' gestation (42). Therefore, they cannot immediately tolerate oral feeds or digest sufficient fortified breast milk to meet nutritional requirements meaning they depend on PN for a bridging period whilst full enteral feeding is safely established.

While PN provision was once concomitant with enteral starvation, this practice is now avoided. Instead, infants are given small milk volumes alongside PN, a technique called trophic feeding (TF) that facilitates a smooth transition from parenteral to enteral nutrition. As the infant's gut matures and enteral feed tolerance improves, oral feeds are advanced using expressed breast milk (EBM) or infant formula. Benefits of TF include stimulation and maturation of neonatal digestive function, a lower incidence of cholestasis and improved feeding tolerance (43,44). While several studies have shown several benefits to TF, the meta-analysis did not confirm the results (45). Furthermore, most clinicians take a cautious approach to advancing enteral feeds due to concerns over developing necrotising enterocolitis (NEC), a life-threatening cause of inflammatory bowel necrosis almost exclusively affecting premature neonates (46). However, the same metanalysis did not suggest an increased incidence of NEC (45). Overall, it is clear that whilst an effective interim measure, PN will never truly replicate the benefits of human breast milk.

1.3.2. History of neonatal PN

PN infusions became available for routine use in the neonatal intensive care unit (NICU) during the 1970s and are established today as part of the standard care package for a VPN. However, the concept of PN was championed and tested long before its first successful use. Table 1.1 briefly summarises key events in the development of neonatal PN.

Year	Accomplishment	Investigators
Early 1900s	1911 - Postoperative use of IV glucose infusions in humans for nutrition	Kausch
	1913 - The first study of IV administered protein was performed in goats using beef protein hydrolysates	Herriques & Anderson
	1924 - First continuous IV glucose infusion in humans	Matas
1930s	1938 - Identification of the EAA and their requirements in humans	Rose
	1939 - First report of successful use of TPN in humans	Elman & Weiner
1940s	1940 - Demonstrated use of a crystalline AA solution in humans	Shohl, Blackfan & Dennis
	1944 - The first documented report of successful complete parenteral feeding (water, saline, fat, carbohydrate, AA) for 5 days in a 5-month-old infant with Hirschsprung's disease	Helfrick & Abelson
	1945 - Development of first polyethene catheters for IV infusions in humans	Zimmerman
	1945 - IV infusion of lipid emulsion, dextrose, and protein hydrolysate through a peripheral vein in humans	McKibbin, Hegsted & Stare
	1947 – First IV protein hydrolysate available commercially in Europe	Wretlind
1950s	1956 - Demonstration that the IV infusion of plasma protein in dogs fed a protein-free diet supported growth	Allen, Stemmer & Head
1960s	1961 - Development of the first safe and efficacious lipid emulsion. Marketed as Intralipid, this remains widely used in PN solutions today.	Schuberth & Wretlind
	1964 - Introduction of the first crystalline AA solution in Germany	
	1967 - Infraclavicular, percutaneous subclavian catheterisation for central venous pressure monitoring in humans	Mogil, DeLaurentis & Rosemond
	1968 - First documentation of normal growth and development in an infant nourished entirely by central TPN	Dudrick & Wilmore
	1968 - First comprehensive technique for long-term total parenteral nutrition in human adults and infants	Dudrick, Wilmore, Vars & Rhoads
1970s	• Crystalline AA solutions replaced protein hydrolysates • All-in-one (AIO) system introduced	Solassol
1980s	1983 - Introduction of long-line silastic central venous catheters (CVC) to provide total parenteral nutrition to neonates	Loeff

Year	Accomplishment	Investigators
1990s	• High and potentially toxic levels of phenylalanine led to a formulation change. • Vamin Infant, a new solution based on the profile of breast milk, entered the market • Studies investigated the efficacy of acetylated forms of tyrosine and cysteine in PN formulations.	Mitton et al. Van Goudoever et al.
2000s	• Various parenteral AA formulations such as Vaminolact (Fresenius Kabi, Sweden) and Primene (Baxter, UK) were developed specifically for neonates. • Research began focusing on individual AA supplementation, particularly glutamine and cysteine, which are unstable in solution.	
2010s	• PN research shifted to finer details, including the timing of initiation, optimum intakes and the transition from PN with enteral nutrition.	

Table 1.1: Key events in the development of neonatal PN (47–49)

Unfortunately, despite modern advances in neonatal care, poor extrauterine growth continues to be expected in this population. Therefore, optimising PN is essential to ensuring the provision of sufficient nutrients and energy since PN is the primary nutrient source for the first two weeks of life.

1.3.3. Complications of neonatal PN

A myriad of complications has been reported associated with older PN solutions. However, with the contemporary formulations, most of these are now rare. Current manufacturing policies promote a high standard of sterility and stability, meaning patients are provided with safe and efficient PN solutions. Nevertheless, PN can still result in mechanical, metabolic, gastrointestinal, and infectious complications during administration (Table 1.2). The primary complications of modern PN are cholestasis and complications related to central lines.

Metabolic Complications	Systemic Complications
Hyperglycaemia	Parenteral nutrition-associated liver disease (PNALD)
Metabolic acidosis	Metabolic bone disease
Hypophosphatemia and other electrolyte imbalance	
Hyperlipidaemia	
Mechanical complications	**Infectious complications**
Extravasation and tissue necrosis	Bacterial infection, especially staphylococcal species
Infiltration	Fungal infections: Candida
Thrombosis	
Pleural or pericardial effusion	
Cardiac arrhythmia from malposition of the catheter	

Table 1.2: Complications of Parenteral Nutrition. Adapted from Mundy and Bhatia (50)

Ideally, PN should be administered via a central line, especially when the patient is expected to be PN-dependent for a prolonged period. The high osmolarity of PN solutions is known to cause peripheral vein thrombophlebitis (51,52). In neonates, PN requires a properly sited peripheral catheter, an umbilical venous catheter (UVC) or a peripherally inserted central catheter (PICC) (4). A designated line should be used exclusively for PN, although, in the NICU setting, this is not always practical.

Infection, particularly central-line associated bloodstream infection (CLABSI), is a significant cause of mortality associated with prolonged IV access. In preterm infants, CLABSI is estimated to cause 70% of all hospital-acquired bloodstream infections (53). However, research has proven that CLABSI is largely preventable by implementing strategies to improve site care, line insertion technique, and training for staff and parents (54).

The numerous complications of PN (outlined in Table 1.2) indicate that it should be administered only when necessary and for the shortest period possible. NEC risk is often a driver for delaying enteral feeds. However, the opposing risk is the development of infection and other PN-related complications due to prolonged PN dependency.

1.4. Amino Acids

The word protein is derived from the Greek word proteious, meaning "of primary quantity", because proteins are the body's major structural and functional components. They serve as precursors for many biologically active proteins, including enzymes, immunoglobulins (Ig), and cytokines, which regulate vital processes in the body. Amino acids (AA) are the building blocks of protein and the driving force for growth. Proteins consist of variable chains of AA monomers joined by peptide bonds, the order of which is defined by the translation of DNA. Parenteral protein intake is provided by crystalline AA solutions, comprising around 20 individual AA.

AA can be classified from a nutritional perspective based on the body's ability to synthesise a particular AA (Table 1.3). An essential amino acid (EAA) cannot be synthesised by the body and must be provided by the diet. The word "essential" does not imply that the remaining non-essential AA (NEAA) are not required for protein synthesis; instead, the others are not essential in the diet because they can be synthesised de-novo by the body. A sufficient supply of all NEAA is required to maintain AA balance. Otherwise, EAA supply is diverted toward NEAA production, away from protein synthesis. In addition, some AA are considered conditionally essential (CEAA), meaning their synthesis can be limited under certain conditions. In the fetus and preterm neonate, the metabolic pathways are underdeveloped and unable to synthesise adequate amounts of certain NEAA. Therefore, this AA group becomes temporarily essential and requires supplementation (55).

The rate of protein synthesis determines the requirements for total AA intake. However, a significant deficiency of a single AA has the potential to impair growth and potentially cause high levels of other AA, which are underutilised. Thus, to achieve optimal growth, the quality of AA supply is equally as important as the quantity supplied.

Essential amino acids (EAA)	Non-essential amino acids (NEAA)	Conditionally essential amino acids (CEAA)
Histidine (His)	Alanine (Ala)	Arginine (Arg)
Isoleucine (Ile)	Asparagine (Asn)	Glycine (Gly)
Leucine (Leu)	Aspartic acid (Asp)	Proline (Pro)
Lysine (Lys)	Glutamic acid (Glu)	Tyrosine (Tyr)
Methionine (Met)	Serine (Ser)	Cysteine (Cys)
Phenylalanine (Phe)		Glutamine (Gln)
Threonine (Thr)		
Tryptophan (Trp)		
Valine (Val)		

Table 1.3: Essential, Non-Essential and Conditionally Essential AA in preterm infants

Unfortunately, any additional regulatory roles for AA have long been ignored. A growing body of evidence has developed the concept of functional AA, that certain AA regulate key metabolic pathways and have important roles in regulating gene expression, cell signalling, antioxidative responses, reproduction, growth and immunity (56). Glutamate, glutamine, and aspartate are major metabolic fuels for the small intestine, and they, alongside glycine, regulate neurological function (57). The deficiency of a functional AA (either EAA or NEAA) can impair protein synthesis and have a knock-on effect on whole-body homeostasis.

There is no storage pool for individual AA in the body, unlike glucose which can be stored in the form of glycogen or fatty acids, stored as adipose tissue. Therefore, surplus individual AA cannot be held after protein synthesis requirements are met. Neither can the kidneys selectively excrete or reabsorb an individual AA. Consequently, excess AA are degraded to ammonia and later converted into urea to avoid aminoacidemia (58). Muscle protein is catabolised when AA or energy supply is inadequate, resulting in muscle atrophy. AA arising from protein breakdown are combined with those obtained from the diet to form the so-called “nitrogen or amino acid pool”, an amalgamation of all the AA available synthesis of new proteins or nitrogen-containing non-protein molecules and energy production (Figure 1.1).

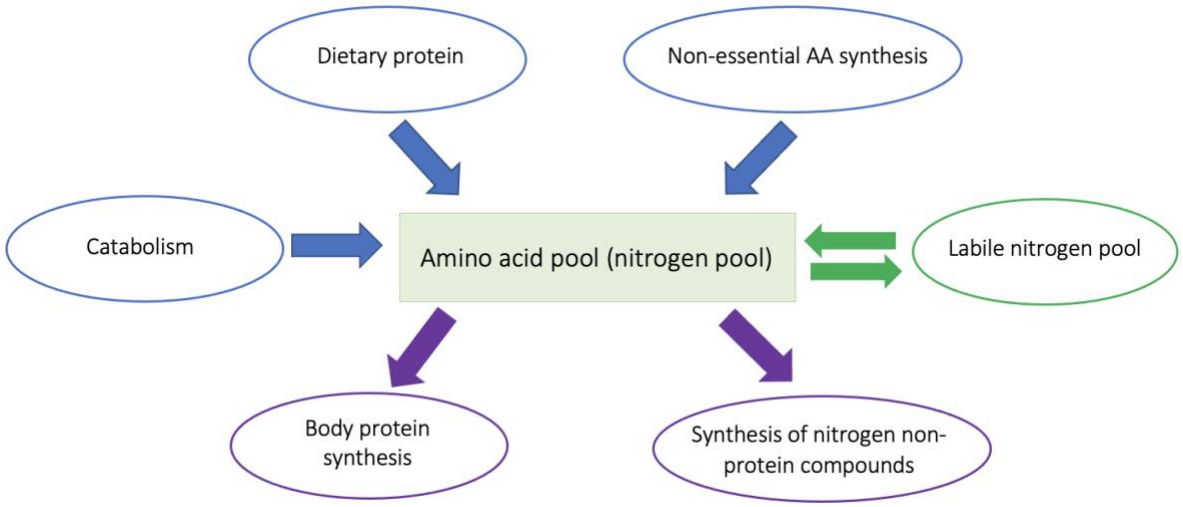


Figure 1.1: Inputs and outputs of the amino acid pool. Adapted from Iacone et al. (58)

Defining the optimal AA formulation for parenterally fed neonates remains particularly challenging. Current commercial neonatal AA formulations are designed to reflect the AA profiles of human milk protein or umbilical cord blood. There is controversy surrounding the most appropriate plasma reference range for individual AA regarding safety and capability for maximum protein accretion. Some argue that the plasma profile of a healthy, breastfed infant is a more accurate reflection (59,60). Furthermore, the overall AA requirement for parenterally fed infants is lower because the supply bypasses the intestine, but there are considerable differences in the intestinal utilisation of specific AA. Additionally, a number of AA are converted into other AA by the intestinal mucosa or liver upon first-pass metabolism. Bypassing the small intestine lowers the bioavailability of those AA, increasing the parenteral requirements.

Overall, individual AA requirements are poorly defined, making it impossible to determine the ideal AA composition for optimal neonatal growth. Consensus is required on the most appropriate reference ranges to determine the best balance of EAA, NEAA and CEAA for the preterm infant, taking into account functional AA requirements.

1.4.1. Importance of parenteral amino acids

VPN receiving only IV glucose catabolises 1.2 g/kg/d of endogenous proteins, reflecting high protein breakdown rates relative to protein synthesis rates (27). The provision of parenteral AA increases protein synthesis, thus reducing the disparity between the rate of proteolysis and protein synthesis. Nitrogen (N) balance is commonly used as a proxy measure of protein balance, assessing whether the quantity of protein (or AA) provided is sufficient to prevent net protein losses. N balance is calculated as the difference between N intake and N losses in urine, stool, skin and other bodily fluids (61). Providing as little as 1.1-1.5 g/kg/d of AA and 30 kcal/kg/d of energy reverses nitrogen balance from substantially negative to zero or slightly positive, minimising the deficit (62,63).

Efforts to reduce the protein deficit are crucial given that the neonatal period is a time of rapid growth and a critical stage of development. In VPN, early growth failure is well described by Ehrenkranz et al., who produced growth curves based on gestational age and birth weight for infants born before 30 weeks (64). The curves showed that most infants born between 24 to 29 weeks' gestation do not achieve the median birth weight of the reference fetus of the same postmenstrual age, and many remain below the 10th percentile at hospital discharge. Most growth failure occurs during the first few weeks after birth, a period of PN dependency, with infants born <1000g taking 14.4 – 17.2 days to regain birth weight. However, interpreting early postnatal weight loss is complicated by physiological postnatal fluid loss (65).

Postnatal growth failure is linked with impaired long-term growth and neurodevelopmental delays, impacting adulthood. The risk of significant neurocognitive disability is well recognised amongst preterm survivors, particularly infants born before 26 weeks (66). Occipitofrontal head circumference (OFC) is an essential anthropometric measure of postnatal growth failure because it correlates with brain growth (67,68) and is associated with general IQ. The fastest period of human brain growth occurs during the last trimester of pregnancy and the first three months of life. VPN navigate this critical period of development ex-utero, exposed to malnutrition and other growth failure risks.

Numerous studies have demonstrated that inadequate provision of early nutrition adversely influences postnatal head growth and long-term developmental outcomes. Studies by Hack et al. revealed that subnormal HC at 8 months was predictive of poorer verbal and performance IQ scores at 3 (69) and 8 years (70). Even at school age, children born prematurely are often smaller and lighter and have a smaller HC than their peers (71,72). More recent studies have produced similar findings correlating improved head growth with better neurodevelopmental outcomes (NDOs) at 2 years (68,73) and 5 years (74,75).

Overall, the data is convincing that there is a potential for undernutrition to cause permanent impairment of central nervous system (CNS) development in preterm infants. Animal studies have shown that malnutrition during a vulnerable period of brain development can decrease brain volume and result in a reduced number of neurons, synapses, dendrites and reactive zones (76). Results of these studies strongly suggest that inadequate nutrition during critical periods of neonatal brain development can alter growth trajectory with adverse neurodevelopmental consequences (77–80). However, the degree and the duration of undernutrition that places infants at risk remains uncertain. Other factors, including illness severity and comorbidity (81), especially chronic lung disease (CLD) (82,83), may have a more potent influence. Nevertheless, the probable benefits of minimising energy and protein deficits should not be underestimated.

Additionally, some studies reported beneficial effects of early or high AA supplementation on the incidence of bronchopulmonary dysplasia (BPD) (84,85) and retinopathy of prematurity (ROP) (84), whilst others did not (32,86). Further benefits of higher parenteral AA include greater synthesis of hormones and enzymes and the improved regulation of oncotic pressure (87). In animal studies, renal hypertrophy and increased circulating insulin-like growth factor-1 (IGF-1) were associated with higher protein intake (88,89).

1.4.2. Current amino acid strategies

Guidelines, such as those presented in 1977 by the American Academy of Paediatrics, continue to stress the importance of parenteral AA for VPN (21). Today, the objective remains the same, to achieve postnatal growth similar to fetal growth rates (4,90,91). The latest guidelines from the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) recommend that for preterm neonates, parenteral AA supplementation should start on the first postnatal day with a minimum intake of 1.5 g/kg/d to achieve an anabolic state that promotes growth. From the second postnatal day onwards, intake should be increased to 2.5-3.5 g/kg/d to ensure growth rates closely match those in-utero (91).

In research terms, the optimal intake of parenteral AA for preterm and term neonates remains controversial (23). Controversies include the optimal starting and incremental doses of parenteral AA (92–94) and ideal final target intake (including actual intake versus prescribed) (95–97). In early PN studies, AA were not initiated until the end of the first week in the smallest infants; neonates depended on glucose infusion during the intervening period (98,99). Following the development of solutions designed specifically for neonates, researchers began investigating the effects of higher AA doses and earlier introduction of PN.

The Cochrane review of early AA (administered in the first 24 h) versus late AA in preterm infants included seven randomised control trials (RCT). The study concluded that there were no apparent benefits of early AA administration on mortality, growth and neurodevelopment. However, early administration of AA did result in positive nitrogen balance, improved acid-base balance and ammonia levels remained normal (92).

Recent studies show that a protein dose of 3.5 or even 4.0 g/kg/d is well tolerated by VPN (85,94,100) despite concerns about metabolic derangement. Results from studies investigating the feasibility of rapidly introduced, high AA intakes were mixed; some showed improved growth and NDOs associated with early and high AA supplementation, while others found no significant differences. A summary of these studies and their findings are provided in Table 1.4.

Author	Study Size	Intended AA intake (g/kg/d)	Key findings
Wilson et al. (1997) (101)	n = 64	0.5 g/kg/d of AA started at 12 hours, increased by 0.5 g/kg/d to a maximum of 3.5 (Early)	Growth in early life and at discharge was significantly improved in babies in the early AA group.
	n = 61	1 g/kg/d of AA started on day 3 and increased by 0.5 g/kg/d to a maximum of 2.5 g/kg/d (Late)	
Poindexter et al. (2006) (86)	n = 182	≥ 3g/kg/d within the first 5 days (Early)	Early AA were associated with significantly better growth outcomes at 36 weeks. At 18 months of corrected gestational age (CGA), the groups had no differences in weight, length, or NDOs.
	n = 836	< 3g/kg/d within the first 5 days (Late)	
Clark et al. (2007) (102)	n = 64	3.5	Higher doses of AA did not improve neonatal growth and were associated with increased blood AA and blood urea nitrogen (BUN) levels.
	n = 58	2.5	
Tan et al. (2008) (22,103)	n = 55	4.0	No significant difference in OFC or other growth parameters at 36 weeks between groups. No difference in NDO at 3 and 9 months post-term.
	n = 59	3.0	
Blanco et al. (2012) (104)	n = 30	4.0 (Rapid)	Early and high AA regimen did not improve growth or NDOs at 2 years. Serum BUN and urine urea were significantly higher in the early and high AA group.
	n = 31	3.0 (Slow)	
Balasubramanian et al. (2013) (105)	n = 75	4.0 (Rapid)	Changes in weight gain, length and head circumference at 28 days were significantly lower in the higher AA group.
	n = 75	4.0 (Slow)	
Burattini et al. (2013) (106)	n = 56	4.0	Body weight, length, and head circumference (HC) at 36 weeks and 2 years were similar between groups. No significant difference in NDOs at 2 years. The higher AA group reported a higher BUN.
	n = 58	2.5	

Author	Study Size	Intended AA intake (g/kg/d)	Key findings
Scattolin et al. (2013) (107)	n = 60	4.0	Significant differences in weight and length at 36 weeks. Weight gain and growth rate during the 3rd week were significantly better in the higher AA group.
	n = 55	3.0	
Vlaardingerbroek et al. (2013) (94)	n = 47	3.6	No significant difference in weight gain, head circumference gain, and knemometry.
	n = 49	2.4	
Morgan et al. (2014) (97)	n = 74	3.8	Higher AA intake (SCAMP nutrition) reported greater HC at 28 days. Differences were still apparent at 36 weeks CGA.
	n = 76	2.8	
Bellagamba et al. (2016) (108)	n = 82	3.5	No difference in weight gain, growth parameters or NDOs at 2 years. Blood urea was higher in the high AA group.
	n = 82	2.5	
Uthaya et al. (2016) (109)	n = 82	3.6 (Immediate)	Head circumference at term was significantly smaller in the high, immediate AA group. No difference in brain volume, weight gain or other growth parameters.
	n = 83	2.7 (Slow)	
Balakrishnan et al. (2017) (110)	n = 85	4.0 (Immediate)	No differences in NDOs were detected between groups. Infants in the high AA group had significantly lower mean weight, length, and head circumference percentiles at 36 weeks CGA and discharge.
	n = 83	4.0 (Slow)	

Table 1.4: Key findings from previous RCTs comparing different parenteral AA strategies

Inconsistent study findings mean a consensus has not been reached regarding the optimal dose of parenteral AA, and it remains uncertain whether higher intakes improve growth at all. There is little assessment of the long-term impact on efficacy and safety, making reliable recommendations difficult. Given the relative lack of high-quality RCTs, current parenteral AA

practice is largely based on uncontrolled or observational studies, expert opinion, and clinical practice guidelines provided by various academic societies, e.g. ESPGHAN.

Finally, there is also significant debate surrounding the appropriate supply of non-protein energy required to utilise AA for adequate growth and metabolism. A minimum of 100-135 kcal/kg/d of energy is required to prevent protein from being used as an alternative energy source rather than for accretion (20). Estimates often do not account for comorbidities that increase requirements. Increasing protein intake without sufficient non-protein energy could result in adverse growth outcomes and increased blood urea (102). However, the glucose and lipid infusion rates required to supply optimal non-protein energy may not be tolerated by the infant, especially during the first week, leading to hyperglycaemia and hyperlipidaemia (23). Clinically, the former could be prevented by reducing glucose intake but is effectively and routinely managed with an insulin infusion (111), although the long-term risks and benefits of this approach are still unknown (112). Additionally, it is difficult for most VLBW infants to reach suggested caloric and protein intakes because of fluid restriction. These difficulties cause frequent interruptions, hindering the advancement of enteral feeding and increasing the risk of PN complications.

There is currently general agreement amongst nutritionists and neonatologists that early initiation of parenteral AA and energy is critical to minimise postnatal growth failure, which is reflected in the guidelines (4,90,113). Nevertheless, the National Confidential Enquiry into Patient Outcome and Death (NCEPOD) enquiry into neonatal PN highlighted huge variation in the quality of PN and nutritional practice throughout the United Kingdom (UK), many of which were considered substandard (114). The monitoring of PN was deemed inadequate in a fifth of infants. PN commencement was delayed in 45% of cases reviewed because the need was unrecognised or not acted upon (115). Despite the guidelines' clarity, these practice differences have been repeatedly identified in UK national PN surveys (116–118). This may partly reflect the wide range of target doses and uncertainty of the topic. However, there is a clear need to review current clinical nutritional practice nationally to improve quality in this fundamental aspect of UK neonatal care.

1.4.3. Problems associated with the provision of AA to preterm infants

The evolution of neonatal PN began in the late 1960s, using the AA composition of cord blood or human milk protein as a start point. The first generation of AA solutions contained protein hydrolysates, produced by heating protein with acid or proteolytic enzymes, followed by purification. These solutions caused significant problems, including hyperammonaemia (119) and were later replaced by the first synthetic crystalline solutions, which caused other issues, particularly acidosis (120). Reporting adverse effects created a fear of metabolic derangement caused by AA, which still influences clinical practice. Following formulation changes, more recent evidence suggests that starting parenteral AA immediately after birth achieves a positive nitrogen balance in the first week of life, improves short-term outcomes, and can be introduced without metabolic complications (121,122), even amongst critically ill infants (123). Nevertheless, some neonatologists are reluctant to adopt the 'early and aggressive' approach to AA administration following dated reports of harmful side effects.

As explained previously, significant progress has been made to improve the safety of PN solutions. However, the commercial PN solutions in use today have not been changed for more than 30 years. This is despite a considerable shift in neonatal demographics and a longstanding body of evidence to suggest the plasma AA profile of PN-dependent VPN is suboptimal. Morgan and Burgess showed that the mean plasma concentrations of EAA are 50-100% above the proposed reference range for healthy preterm infants receiving Vaminolact, a solution modelled on the AA composition of breast milk (124). On the other hand, mean plasma concentrations of CEAA are up to 50% lower than the reference range. The same AA imbalance is seen in Primene (125), another commonly used PN AA solution in the UK modelled on the AA composition of cord blood, and TrophAmine (126), a formulation widely used in the United States. Practical reasons explain particular AA deficiencies, including solubility in the case of tyrosine and stability for cysteine and glutamine. However, arginine is soluble and stable in PN solutions, and hypoarginemia is a consistent finding.

Furthermore, merely increasing AA supply does not prevent the deficiency of CEAAs in PN-dependent VPNs (124). The consistency of the imbalance is concerning because a single AA deficiency could undermine the high AA intake strategies used to promote growth.

There have also been concerns about the long-term health effects of aggressive nutrition strategy in VPN. In the 1970s, reports began linking adverse nutritional provision in early life with late-onset disease, particularly coronary heart disease. David Barker developed this idea of "nutritional imprinting". He proposed that metabolic syndrome and cardiovascular disease may partly result from metabolic programming in-utero or during early postnatal life (127–129). The association with long-term disease is thought to be a consequence of developmental plasticity, whereby the environmental conditions during development can permanently alter gene expression through epigenetics. However, the development of late-onset disease is multifactorial. Until further evidence proves that early nutritional intervention causes more harm than benefit, there is no reason to change current PN strategies, especially given that the first few weeks are a crucial period of neurodevelopment.

To summarise, despite the extensive use of PN, progress in the field of nutrition is limited compared with other developments in neonatology, such as mechanical ventilation. Even with the current focus on “early and aggressive” nutrition in the NICU, undernutrition and postnatal growth failure remain important problems for VPN. Vast uncertainty remains surrounding optimal intakes of parenteral AA and the ideal composition of neonatal AA solutions. Consequently, new nutritional strategies should be explored, and attempts must be made to remodel the composition of AA solutions to meet the needs of VPN and improve the plasma AA profile.

1.5. Infection in the neonatal period

Neonatal infection and sepsis are prominent causes of paediatric morbidity and mortality. Sepsis is a dysregulated host response to infection leading to life-threatening organ dysfunction (130). Unlike mortality associated with respiratory distress, which has shown a constant decline, mortality from neonatal infections in preterm infants has increased during the last 20 years (131). The 2016/17 Global Burden of Disease (GBD) Study estimated there were 1.3 million cases of neonatal sepsis worldwide annually, resulting in 203 000 sepsis-attributable deaths (132). However, important contributors to the burden of neonatal infection and sepsis, including pneumonia, are not captured by the GBD estimate. Neonates are disproportionately affected in low-income countries with a high prevalence of infectious diseases and poorer access to healthcare facilities. Hence, neonatal sepsis remains a commonly encountered and feared complication worldwide, especially for preterm infants.

Neonatal sepsis is divided into early-onset sepsis (EOS) and late-onset sepsis (LOS) based on the time of onset and mode of acquisition. There is disagreement regarding the exact cut-off between early and late-onset. Some sources define EOS as infection within the first seven days, and others limit it to infections within the first 72 hours. EOS is likely due to the intrapartum vertical transmission of organisms from the mother. The commonly implicated organisms are Group B Streptococcus (GBS) and Escherichia coli, accounting for approximately 70% of infections combined (133). LOS, in contrast, is attributed to postnatal pathogen exposure (134). The different organisms associated with EOS and LOS are summarised in Table 1.5. For VPN, invasive procedures and devices, especially long-term IV catheters used for PN, result in ongoing infection risk. The other risk factors for neonatal sepsis are summarised in Table 1.6.

Early-onset sepsis	Late-onset sepsis
Group B Streptococcus	Coagulase-negative staphylococci
Escherichia coli	Staphylococcus aureus
Enterococci	Enterococci
Other streptococci: S.pyognes, S.viridans, S.pneumonia	Multi drug-resistant Gram-negative rods (E.coli, Klebsiella, pseudomonas)
Haemophilus influenza	Candida

Table 1.5: Major microbial causes of neonatal sepsis (135,136)

Early-onset sepsis	Late-onset sepsis
Maternal GBS colonisation	Breakage of the natural barriers (skin and mucosa)
Chorioamnionitis	Prolonged indwelling catheter
Prolonged rupture of membranes (>18 hours)	Invasive procedures (e.g. Endotracheal intubation)
Premature rupture of membranes	Necrotising Enterocolitis
Maternal urinary tract infection	H2-receptor blocker or proton pump inhibitor use
Multiple pregnancy	
Procedures during pregnancy (amniocentesis, cervical cerclage)	

Table 1.6: Risk factors for neonatal sepsis (137)

Early diagnosis of neonatal sepsis is difficult because the clinical manifestations are subtle, non-specific and may also be associated with prematurity or the normal physiological transition to extrauterine life (Table 1.7). In a cohort of 240 neonates with sepsis risk factors, only 2 out of the 12 patients with true-positive blood cultures (BC) exhibited signs and symptoms of sepsis. The remaining 10 infants were asymptomatic at the time of admission, calling attention to the challenge of early identification of septic neonates (138).

Category	Signs
Respiratory	Respiratory distress (tachypnoea, nasal flaring, grunting, recessions) Hypoxaemia requiring increased supplemental oxygen
Circulation	Tachycardia Hypotension Delayed capillary refill Diminished pulses
Gastrointestinal	Reduced feeding or alterations in feeding pattern Vomiting Abdominal distension
Neurological	Hypotonia or hypertonia Seizures Bulging fontanelles
Dermatological	Mottled or ashen appearance Non-blanching rash Cyanosis of skin, lips or tongue
Behavioural	Weak, high-pitched, or continuous cry Irritability Lethargy
Other	Temperature abnormality (lower than 36°C or higher than 38°C) Jaundice in the absence of other risk factors for hyperbilirubinemia Altered glucose homeostasis (hypoglycaemia or hyperglycaemia)

Table 1.7: Clinical indicators of possible neonatal infection (139)

The gold standard for diagnosis is a positive BC result, which is limited in sensitivity by intrapartum antibiotic administration and constraints in blood volume per culture that can be safely collected in VPN (140). Furthermore, decisions about antibiotic management are affected by the 48 -72 hour turnaround time for BC results.

Typical management of neonatal sepsis is broad-spectrum IV antibiotics that are narrowed down once the pathogen and its antibiotic sensitivity are confirmed. Common initial empirical antibiotic combinations for EOS are benzylpenicillin with gentamicin or flucloxacillin plus gentamicin to cover the causes of LOS (139).

Neonates, especially preterm infants, are vulnerable to infection for two main reasons: deficits of the immune system subsequent to prematurity and continual medical interventions which interfere with protective mucosal and epithelial barriers (mechanical ventilation and intravenous lines). The impact of prematurity on the developing immune system is discussed further in Chapter 4 of this Thesis.

Preterm infants who survive neonatal infection may experience permanent disability due to organ damage caused by the infection itself or the inflammatory response generated by oxidative stress (141). Neonatal infection can result in meningitis which carries a significant risk of permanent neurological impairment (142). Evidently, neonatal infections pose important healthcare challenges, and the administration process of PN increases infection risk. Therefore, the length of PN dependence should be carefully considered to reduce the burden of infection amongst VPN.

1.6. Evidence from critically ill adult and paediatric populations

A recent, large multicentre RCT (EPaNIC) unexpectedly revealed in a population of critically ill adults that withholding PN for seven days resulted in an increased likelihood of an earlier live discharge from the Intensive Care Unit (ICU) with fewer complications (143). A similar multicentre study was later conducted on 1440 critically ill children from term newborn to 17 years of age (PEPaNIC). It was reported that the children who received late PN had a lower odds of an infection, a shorter period of dependency on mechanical ventilation and a reduced Paediatric ICU (PICU) stay (6.5 ± 0.4 days versus 9.2 ± 0.8 days). The improved short-term benefits from withholding PN in children were larger than those seen in adults (144).

Considering statistical power, the benefits of withholding PN during critical illness occurred for term neonates in agreement with findings from older children and adults (145). Delayed introduction of PN reduced the risk of nosocomial infections amongst term neonates aged 0 - 7 days (adjusted odds ratio = 0·36, 95% CI = 0·15-0·83, p = 0.017). The study also found that higher doses of AA, rather than glucose or lipid, were associated with a prolonged need for intensive care and an extended period of dependency on mechanical ventilation. These findings should be interpreted with caution, given that the infants studied were born at term gestation and had vastly different nutrition requirements compared with VPN.

However, another RCT investigating the growth benefits of high-dose, early AA supplementation was halted after the inclusion of 50 infants due to a concerning incidence of LOS in the intervention group (146). The authors attributed this to uncorrected hypophosphatemia resulting from phosphate depletion due to accelerated protein synthesis, a recognised metabolic consequence of AA supplementation. This evidence supports the notion that aggressive PN strategies may require additional electrolyte supplementation. These findings indicate that the PEPaNIC findings may extend to the preterm population, which renewed concerns about early AA provision to VPN, the paediatric population forming the bulk of patients receiving PN.

Together the EPaNIC and PEPaNIC trials suggested that in critically ill patients, withholding PN for the first seven days was clinically superior compared with early initiation of PN. The benefits were seen even in term neonates <1 day and those unable to tolerate minimal enteral nutrition, similar to a VPN. However, delaying parenteral AA for as long as a week in preterm neonates contradicts current guidance and is ethically unacceptable. Nevertheless, these findings raise important questions about the presumed benefits of an “early and aggressive” approach to AA supplementation and the risk of infection that require further investigation before changes are made to PN guidelines.

1.7. Autophagy: A fundamental mechanism for cell survival

The underlying mechanisms explaining the improved outcomes following the delayed initiation of PN remain speculative. The PEPaNIC study authors suggested that one plausible explanation for the difference in infection rate may be deficient autophagy following the early initiation of PN (145). Autophagy contributes to basal cellular metabolism and homeostasis, alongside having an emerging role in the innate immune response (147).

1.7.1. Process of autophagy

Autophagy is the major lysosomal mechanism by which cells degrade protein and damaged organelles. The term autophagy is derived from the Greek words "auto", meaning self, and "phagein", meaning to eat (148). Autophagy ensures metabolic homeostasis in all living organisms, from yeast to humans. There are several forms of autophagy; the dominant form is macroautophagy (referred to as autophagy hereafter). During autophagy, a portion of the cytoplasm is surrounded by a membrane derived from specialised regions of the endoplasmic reticulum to form an autophagosome. Upon fusion with a lysosome, the initial autophagosomes acquire lytic enzymes to form autophagolysosomes which degrade sequestered cellular material (149). A series of autophagy-related genes (Atg) have been discovered, which are involved in the formation and maturation of autophagosomes (150,151).

1.7.2. Regulation of autophagy

The major intracellular regulator of autophagy is the nutrient-sensing mammalian target of rapamycin (mTOR) complex 1 (mTORC1). Autophagy is negatively regulated by mTOR. When nutrient supply is abundant, mTOR promotes cell growth and metabolic activity while suppressing autophagy through the phosphorylation of ULK1 (the mammalian ortholog of yeast Atg1) (152). The autophagy pathway is summarised in Figure 1.2. AA stimulate protein synthesis through mTOR, and leucine especially is a potent activator of mTOR.

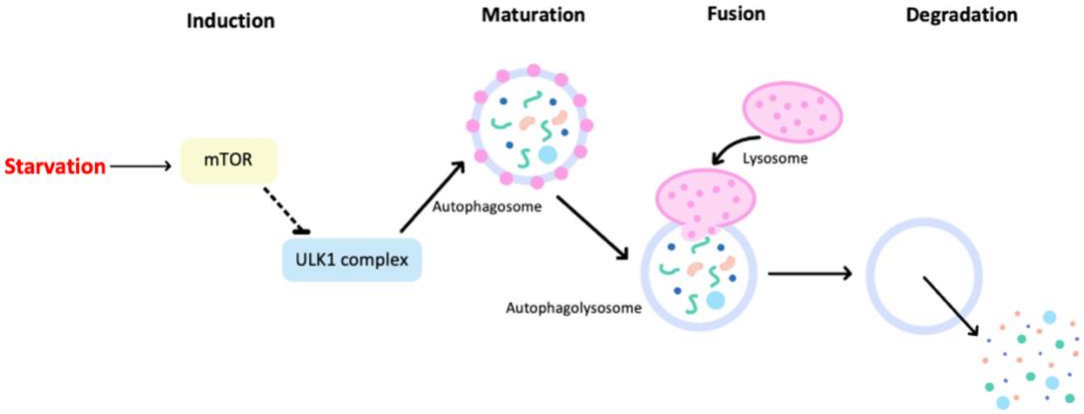


Figure 1.2: The process of autophagy. Autophagy is negatively controlled by mTOR. Autophagosomes fuse with lysosomes to degrade intracellular content and recycle macromolecule components. Figure adapted from N. Chang, Autophagy and Stem Cells: Self Eating for Self-Renewal (152).

However, in response to starvation, protein degradation via autophagy is accelerated to produce AA for gluconeogenesis (153), providing an internal source of nutrients for energy. Rapid and efficient activation of metabolic autophagy following a decrease in nutrient supply appears crucial for cell survival (154). Under homeostatic conditions, cells maintain a basal level of autophagy as a method of routinely turning over cytoplasmic content.

1.7.3. Autophagy in disease

Autophagy prevents the accumulation of damaged proteins and organelles that cause chronic tissue damage and disease. Dysfunctional autophagy has been linked to cardiac dysfunction during periods of starvation (155), liver disease (156), and the development of Crohn's disease (157). In the brain, autophagy prevents the accumulation of ubiquitinated proteins and disposes of aggregation-prone proteins, which cause neurodegeneration in Huntington's and Parkinson's disease (154,158).

Defective autophagy has also been associated with an impaired immune response. Autophagy intersects with nearly all components of the innate immune system, controlling phagocytosis, antigen presentation and cytokine production (148). The relationship between autophagy, infection and the developing immune system is discussed in more detail in Chapter 4. Knowledge of immunological autophagy is still developing, and many important questions

remain. It should be noted that much of the work surrounding the role of autophagy in disease has been done in murine models, meaning it may not reflect mechanisms in human VPN. Regardless, in translational terms, autophagy is becoming an attractive target for disease prevention and treatment of inflammatory disorders (148). Further work is required to test these concepts in the VPN population concerning neonatal infection.

1.7.4. Autophagy in the neonatal period

Autophagy may have a crucial role in the early neonatal period. At birth, the constant placental nutrient supply is suddenly interrupted, and the infant enters a period of starvation whilst nutritional intake, enteral or parenteral, is established. Autophagy is activated during the neonatal starvation period to recycle AA for energy (159). However, other roles have been proposed for autophagy in the neonatal period, including programmed cell remodelling during the fetal-neonatal transition and elimination of residual embryonic structures. Autophagy may also support the response to physiological oxidative stress at birth (160). Clearly, basal levels of autophagy are needed in neonates as part of the transition to neonatal life.

Often, PN is initiated early to reduce muscle catabolism during periods of critical illness. However, given that autophagy is activated by fasting, which includes periods of anorexia induced by critical illness or prematurity, it follows that early initiation of neonatal PN would inhibit autophagy activation (159). Deficient autophagy has previously been documented in humans with a prolonged critical illness (161) and parenterally fed rabbits (162); the severity of the deficiency correlated with AA intake. Meanwhile, autophagy is highly involved in regulating innate immune responses, meaning impaired autophagy (through the provision of parenteral AA) may dampen the neonatal immune system. These findings offer a framework to interpret the apparent clinical benefits of withholding PN in critically ill patients, including term neonates. Given the suppressive effect of AA, including leucine, on autophagy, the role of PN during critical illness (including prematurity) should be investigated further.

1.8. Importance of leucine

Previous work has examined parenteral AA provision as a whole. PEPaNIC and many preterm-specific PN studies have not considered whether the potential overprovision or deficiency of single AA has an effect. As discussed above (1.4. Amino Acids), each individual AA has a role in protein structure, but many have additional regulatory roles in metabolism. Arginine and glutamine, examples of very metabolically active AA, are deficient in parenteral AA formulations. These deficiencies have been linked to potential adverse outcomes (163,164), and efforts are being made to correct deficiencies, but the potential risks of AA overprovision have not been as extensively studied.

This thesis has a particular focus on leucine (Leu), a branched-chain amino acid (BCAA) and one of the nine EAAs, which cannot be synthesised by the body and must be obtained directly from the diet. Parenteral leucine requirements are likely high because leucine appears to be a significant regulator of muscle protein synthesis in neonates (165). Escobar et al. showed that supplementation with parenteral leucine enhanced muscle protein synthesis in neonatal pigs (166). However, studies of plasma AA profiles in preterm baboons (167) and human VPN (124–126) consistently show EAA levels, including leucine, well above the reference ranges for healthy preterm infants, indicating that potentially PN formulations provide an excess of leucine.

Table 1.8 shows the leucine content (g/100g AA) of the three most widely used neonatal AA formulations. The leucine content of human breast milk protein is 9.9 ± 8.4 g/100g AA (168). Despite evidence of overprovision by plasma AA profile data, the leucine content of each AA formulation is within the standard deviation for that of breast milk protein. This indicates no obvious, direct overprovision of leucine by AA solutions themselves. Nevertheless, the imbalance in the plasma AA levels requires further investigation.

AA source	Usage of AA formulation in UK level 3 NICU	Leucine content (g/100g AA)
Vaminolact	83%	10.7
Primene	15%	10.0
TrophAmine	Unlicensed in the UK	14.0

Table 1.8: Leucine content of neonatal parenteral and enteral nutrition sources. Adapted from the PAINT protocol and Morgan et al. (124)

Leucine is also part of the "functional" AA group (57) because of its recognised role as a potent activator of the mTOR pathway (169). mTOR-mediated signalling stimulates postprandial protein synthesis and inhibits catabolic processes, particularly autophagy, by inhibiting autophagosome formation. It is unknown whether overprovision of EAA, especially leucine, to the point of "toxicity", impairs the immune response through reduced autophagy. However, given the recent evidence associating parenteral AA and a higher risk of infection, these additional functional properties make leucine an ideal AA to investigate further and potentially optimise in VPN.

1.9. Justification

PN is a fundamental element of modern neonatal care which influences short and long-term outcomes for VPN. Evidence suggests that existing AA formulations are imbalanced, resulting in high plasma levels of leucine and other EAA. Given the findings of the PEPaNIC study, which associates early initiation of parenteral AA with a higher risk of infection, further research is needed into the effect of EAA overprovision on postnatal immune adaptation. The relationship between individual AA plasma levels and the development of neonatal infection requires investigation. To better meet VPN needs, there may be a need to remodel parenteral AA solutions. This thesis focuses on leucine because of its potent effect on mTOR activation, quantifying the relationship between parenteral leucine intakes with plasma levels in PN-dependent VPNs to determine the optimal leucine content of neonatal PN.

The frame for this thesis is the hypothesis that autophagy, a cellular process important for innate immunity, is deficient in VPN because of EAA overprovision. The thought process behind this hypothesis is summarised in Figure 1.3. The validity of this hypothesis depends on the totality of evidence from several perspectives, not purely neonatal research. Ultimately, it remains unclear whether the excess provision of EAA (including leucine) to the point of "toxicity" can have such an effect that it impairs the immune response through reduced autophagy. This work will introduce the idea of using transcriptomics, the study of RNA transcripts, to start examining postnatal gene expression changes during the first two weeks of life.

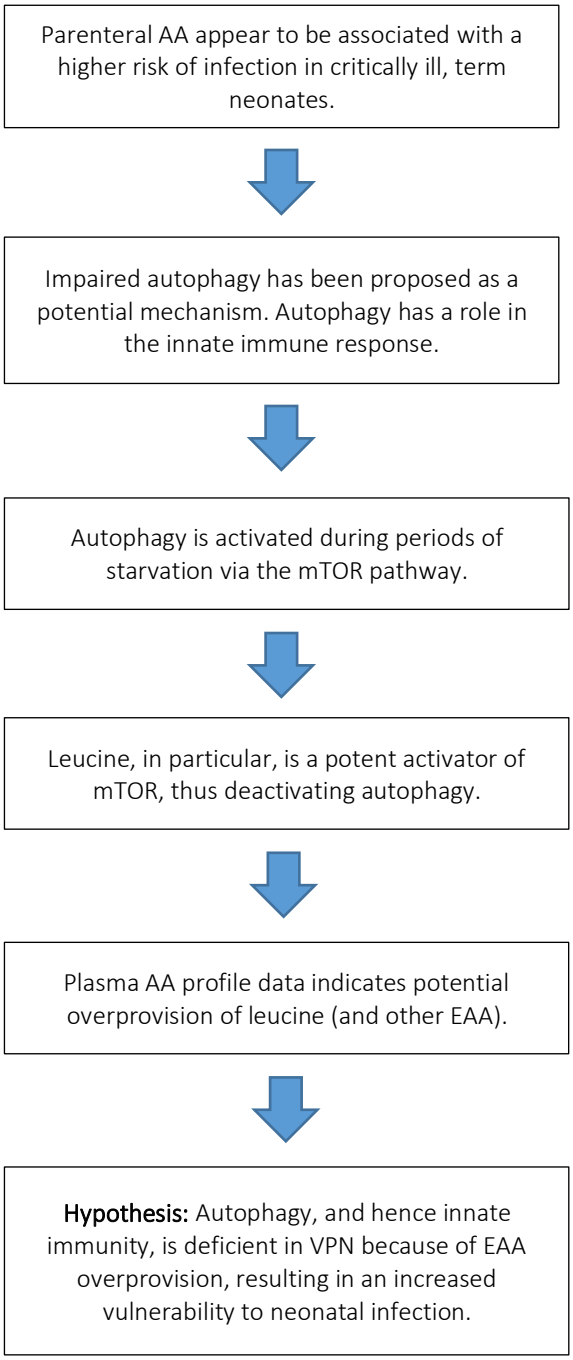


Figure 1.3: Flowchart outlining hypothesis generation

1.10. Aim and objectives of the thesis

The overall aim is to provide an overview of the available methods which could be used to assess the importance of individual parenteral AA and optimise PN. Reviewing these methodological approaches will help understand how modifying AA formulation may impact early postnatal metabolic adaptation. This thesis considers three methodological perspectives, identifying the strengths of each approach and determining what is required to maximise the value of each method when reviewing the hypothesis in detail.

We had the following objectives:

- To investigate the relationships between nutritional intake data, plasma amino acid levels, and infection-related outcomes using existing data from the SCAMP nutrition study (Chapter 2).
- To investigate and quantify the relationship between parenteral leucine intakes and plasma leucine levels by conducting a systematic review (Chapter 3).
- To explore whether conducting a transcriptomic analysis could be used to examine the time course of autophagy-related transcripts, exploring whether autophagy and related pathways change between day three and day ten after birth (Chapter 4).

Chapter 2: Parenteral Nutrition and Neonatal Infection in the Very Preterm Infant. A Secondary Analysis of the SCAMP Nutrition Study.

2.1. Background

The relationship between parenteral AA provision, specifically relating to individual AA plasma levels, and the development of neonatal infection requires investigation. This thesis chapter entails further analysis of the data from the SCAMP study. The analysis first compares total parenteral AA intake and then individual plasma AA concentrations between infants who developed late-onset sepsis (LOS) and infants who did not. Given the proposed link between parenteral AA intake, autophagy deficiency and infection in the PEPaNIC study, we hypothesised that a high AA intake or high plasma leucine might be associated with an increased incidence of LOS and necrotising enterocolitis (NEC) in VPN.

2.2. Methods

The SCAMP study (ISRCTN: 76597892) received ethical and regulatory approval and is described in greater detail with the published primary outcome (97). The study was a single-centre, parallel-group randomised control trial (RCT) with blinding of the caregivers, parents and outcome assessors. Eligible infants were born <29 weeks' gestation and weighed <1200g. They were admitted to the NICU at Liverpool Women's Hospital (LWH) within 48 hours of birth between October 2009 and July 2012. After parental consent, infants were randomised to a SCAMP or control PN regimen. Both regimens used Vaminolact (Fresenius Kabi). Randomisation was required within 120 hours of birth, and the study intervention continued until 28 completed days of life.

The primary objective of the SCAMP study was to investigate postnatal head growth velocity in the first 28 days of life, with the study of infection outcomes as a planned secondary analysis. Infection monitoring, investigation and treatment were in accordance with the LWH guidelines. Patient data were collected from the electronic patient record and recorded in an EXCEL case report form (CRF). The effect of SCAMP nutrition on central venous catheter (CVC) complications, including infection, has already been reported (170). For this analysis, the

infants were re-stratified into two groups, infants who developed LOS and infants who did not. That way, individual AA concentrations could be directly compared between the two infection status groups. LOS was defined as a positive blood culture (BC) after 72 hours of postnatal age.

The infants were later stratified again, expanding the infection group to include infants who developed NEC, an acute inflammatory disease of the intestine and a notable cause of morbidity and mortality in the NICU. A systematic review of recent large cohort studies reported an overall mortality rate of 23.5% in infants with confirmed NEC (Bell 2a or greater). Meta-analysis revealed that extremely low birth weight infants (ELBW, <1000g) with surgical NEC have the greatest mortality rate (50.9%) (171). NEC onset is often within the first three months of life, and ELBW neonates or neonates under 28 weeks' gestation are the most susceptible (172). The pathogenesis of NEC is only partly understood but likely involves a dysfunctional intestinal barrier immune response to enteral nutrition and small bowel bacterial colonisation. Infections are thought to play an important role in the pathogenesis of NEC (173).

To conduct the analysis, Keziah Davies (KD) collected patient data from the study CRFs, including BC results and daily parenteral AA intakes for the first 4 study days. The hourly volume of each component of parenteral and enteral nutrition, fluid and drug infusions is captured by routine nursing charts. Each 'day' (24-hour period) starts at the time of birth. VPN receive protein parenterally or from various enteral sources (human milk, fortifier, and formula milk proteins). For this analysis, we chose to focus only on total parenteral intake, in line with the aims of the thesis. Therefore, we excluded the small contribution of enteral nutrition to total AA intake during the first week of life. On day 4 of life, the mean enteral protein intake was 0.1g/kg/d for the recruited infants.

As part of this analysis, KD retrieved and transcribed the BC results and nutritional intake data for the 9 infants who passed away during the study period from the original paper CRFs. These infants had not been included in the previous infection-outcome analysis by Tan et al. (22). Given that some of the data and nutrition formulae had already been collated into an existing database, KD contributed to the data collection for a similarly designed physiological study, PAINT18, to gain a better understanding of how the SCAMP data had been collected. Involvement is discussed further in Chapter 4.6.

Parenteral “protein” is supplied as a mixed AA formulation, unlike human breast milk protein, which is delivered as whey and casein protein fractions hydrolysed to AA during digestion. In the SCAMP study, total parenteral AA intake (g/kg/d) was calculated using the daily volume of aqueous PN and the manufacturer’s summary of product characteristics (SmPC) document. It is important to note that although the AA composition of the PN formulation is being studied, nutritional intakes are also commonly reported in terms of protein or nitrogen content. Certain AA formulations require a conversion factor to calculate AA intakes. For example, using the SmPC document for Vaminolact (the formulation used by the SCAMP study), the total AA content was 65.3 g/L, whereas the total protein content was 58 g/L (Appendix 1).

Hence the conversion factor used by the manufacturing company was calculated:

Conversion factor of protein to AA for Vaminolact = 65.3 g/L ÷ 58 g/L
= 1.13

For the infants allocated the SCAMP nutrition regimen, this equates to a maximum of 3.8g/kg/d of protein but 4.3g/kg/d of AA. The nitrogen to protein conversion factor can be calculated similarly.

The plasma profile of 20 proteogenic individual AA (µmol/L) was recorded from the first sample plasma AA sample obtained for routine clinical monitoring. Our clinical guidelines recommended this is approximately 7 days after maximum AA intake has been achieved, and only in infants receiving >50% nutrition intake (volume) parenterally. Plasma AA levels were measured using ion-exchange chromatography (IEC) with normal reference ranges obtained from a recent, multicentre U.K. study of infants <6 months old (including infants analysed in our own laboratory) using the same analysis technique (174).

Statistical Analysis

The study was powered according to the primary outcome. First, the Shapiro-Wilk test was used to test for normality. Then, two-sample t-tests were used for normally distributed data, and Mann-Whitney U tests were used for data which was not normally distributed. Chi-squared tests were also used for categoric data. A p-value of ≤ 0.05 will be considered statistically significant.

2.3. Results

The demographic data of the SCAMP (n = 74) and control (n = 76) groups have been reported previously (97). Following stratification based on infection status, 61 infants were found to have a positive BC during the study period, and 89 infants did not. Of the infants that developed an infection, 7 had a single positive BC before 72 hours. By definition, these infants developed early-onset sepsis (EOS). They were analysed as part of the no-infection group because their infection was most likely attributed to intrapartum transmission of organisms and was unlikely to be influenced by AA intake. Therefore, a total of 96 infants were analysed in the no-infection group. The demographics of the new stratified groups are summarised in Table 2.1. Among the VPN who developed an infection, the median (IQR) day of the first positive BC was 11 (8-14).

Demographic factor	Infection - LOS only (n = 54)	No infection (n = 96)	P value
Birth weight, mean (SD), g	839 (134)	921 (182)	0.003
Gestation, mean (SD), w	26.4 (1.4)	26.8 (1.3)	0.11
Gender, n (%) boy	33 (61)	50 (52)	0.29
Number receiving SCAMP nutrition	24	50	0.37

Table 2.1: Comparison of key demographic factors between infection and no-infection groups

The daily parenteral AA intake data were available for all 150 infants. However, eight infants did not have a plasma AA profile performed in the infection group, leaving 46 profiles for analysis. In the no-infection group, 16 infants did not have a plasma AA profile, leaving 80 for analysis. A total of 126 plasma AA profiles are included in the analysis. The median (IQR) day of the first plasma AA sample was day 9 (8-10) of life. The pathway of detecting eligible infants for inclusion in each stage of the analysis is summarised in Figure 2.1.

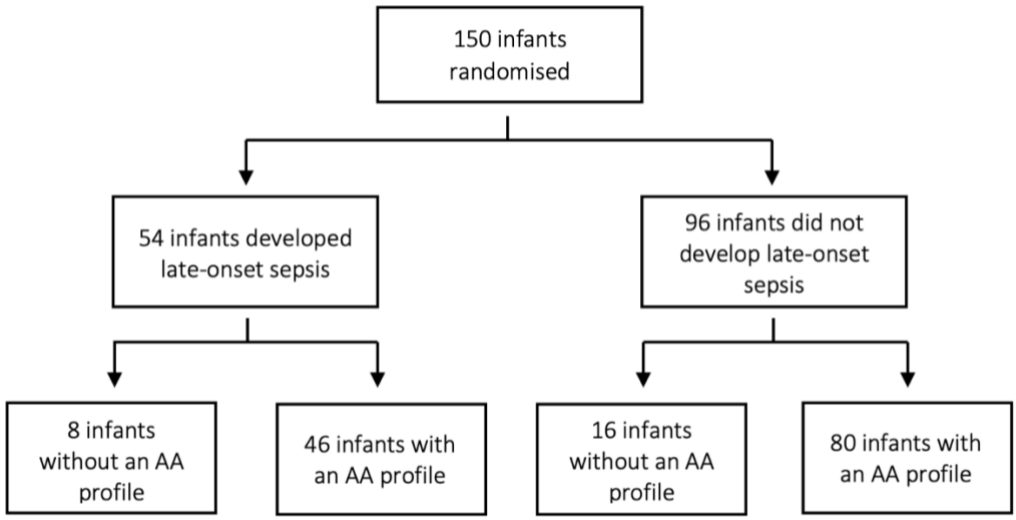


Figure 2.1: Flowchart showing the stratification based on infection outcome and availability of AA profile data for each infant in the SCAMP study

2.3.1. Total parenteral AA Intake

The total parenteral AA intake during the first four postnatal days was calculated for each infant (g/kg/4d). Table 2.2 shows the mean total parenteral intake difference between infants who developed an infection by day 28 and those who did not. The difference in total intake was not significant.

	Mean (SD) total parenteral AA intake by day 4 (g/kg/4d)		P value
	Infection (n = 54)	No Infection (n =96)	
Day 28	7.87 (1.19)	7.89 (1.27)	0.95

Table 2.2: Comparison of total parenteral AA intake between infection and no-infection groups

2.3.2. Individual plasma levels

Table 2.3 compares individual plasma AA levels (µmol/L) between infants who developed LOS during the study period and those who did not. In general, plasma AA levels are lower in the infection group, particularly for the CEAA. There is evidence of EAA overprovision in both groups, with plasma levels above the median reference value. Overprovision is most noticeable for threonine, which is above the upper limit of the reference range. However, after allowing for multiple comparisons using the Bonferroni correction method, there are no significant differences in plasma levels between the groups for any of the individual AA.

Amino Acid	Mean plasma level (µmol/L)		P value	Reference Range [b]
	Infection (n = 46)	No infection (n = 80)		
NEAA				
Ala	341.82	382.87	0.13	300 (112-592)
Asn	30.80	33.95	0.29	38 (18-58)
Asp	33.23	36.89	0.87	19 (17-21)
Glu	97.11	110.67	0.51	100 (32-240)
Ser	243.57	258.53	0.21	127 (69-206)
EAA				
His	90.14	92.23	0.59	74 (43-111)
Ile	48.14	51.24	0.13	50 (20-91)
Leu	148.77	147.25	0.95	97 (44-169)
Lys	234.91	255.33	0.23	155 (70-266)
Met	27.52	32.08	0.05 [a]	25 (11-49)
Phe	80.57	85.20	0.76	52 (25-80)
Thr	534.70	514.77	0.65	97 (39-175)
Try	17.84	20.19	0.06	15 (10-19)
Val	172.41	175.28	0.67	146 (65-290)
CEAA				
Arg	36.48	46.62	0.04 [a]	57 (12-112)
Gly	384.50	414.85	0.18	246 (120-436)
Pro	348.41	361.75	0.98	154 (66-330)
Tyr	52.09	82.84	0.06	58 (22-103)
Cys	25.09	30.20	0.03 [a]	-
Gln	430.16	492.30	0.04 [a]	559 (323-810)

Table 2.3: Comparison of mean individual plasma AA levels between infection and no-infection groups

[a] $P \leq 0.05$; P values not corrected for multiple testing
[b] Median (IQR) reference AA levels from a population of infants <6 months old (174)

Finally, the infection group was expanded to include infants who also developed NEC. 13 infants developed NEC without also having a positive BC result during the 28-day study period. One infant did not have an AA profile, but the remaining 12 were analysed as part of the infection and/or NEC group. Analysis of infants with either an infection or NEC revealed a similar PN imbalance between excess overprovision and CEAA deficiency, particularly for arginine (Table 2.4). However, after correcting for multiple testing, there were no significant differences between the groups.

Amino Acid	Mean plasma level (µmol/L)		P value	Reference Range [b]
	Infection +/- NEC (n = 58)	No infection/NEC (n = 68)		
NEAA				
Ala	354.02	378.01	0.27	300 (112-592)
Asn	34.17	31.72	0.91	38 (18-58)
Asp	33.88	36.93	0.22	19 (17-21)
Glu	107.91	103.97	0.23	100 (32-240)
Ser	243.19	261.63	0.06	127 (69-206)
EAA				
His	91.14	92.37	0.86	74 (43-111)
Ile	48.40	51.44	0.13	50 (20-91)
Leu	147.29	145.85	0.88	97 (44-169)
Lys	250.36	250.10	0.70	155 (70-266)
Met	29.10	31.97	0.16	25 (11-49)
Phe	82.67	84.01	0.72	52 (25-80)
Thr	520.91	524.24	0.88	97 (39-175)
Try	17.60	21.01	0.02 [a]	15 (10-19)
Val	177.36	171.13	0.43	146 (65-290)
CEAA				
Arg	36.03	49.60	0.003 [a]	57 (12-112)
Gly	395.19	412.84	0.25	246 (120-436)
Pro	361.33	352.49	0.41	154 (66-330)
Tyr	64.74	77.12	0.21	58 (22-103)
Cys	26.05	30.82	0.04 [a]	-
Gln	440.83	497.18	0.02 [a]	559 (323-810)

Table 2.4: Comparison of mean individual plasma AA levels between infection and NEC outcome groups

[a] $P \leq 0.05$; P values not corrected for multiple testing
[b] Median (IQR) reference AA levels from a population of infants <6 months old (174)

2.4. Discussion

The study did not find a statistically significant difference in total parenteral AA intake during the first four days of life. Focusing on intake over the first four postnatal days is appropriate for this analysis because parenteral protein (and subsequently parenteral AA) intake (g/kg/d) remained similar between the two regimens (Figure 2.2). Each infant started on standardised, concentrated neonatal PN (scNPN) at birth. After randomisation, patients were switched to a SCAMP regimen or remained on scNPN (control group), an incremental increase which took 2-5 days. Consequently, data from both treatment allocation groups are appropriate for analysis which significantly increases the size of the data set. A second benefit of using day four as the upper limit is that the intake data were available for all 150 participants, including those who passed away during the 28-day study period.

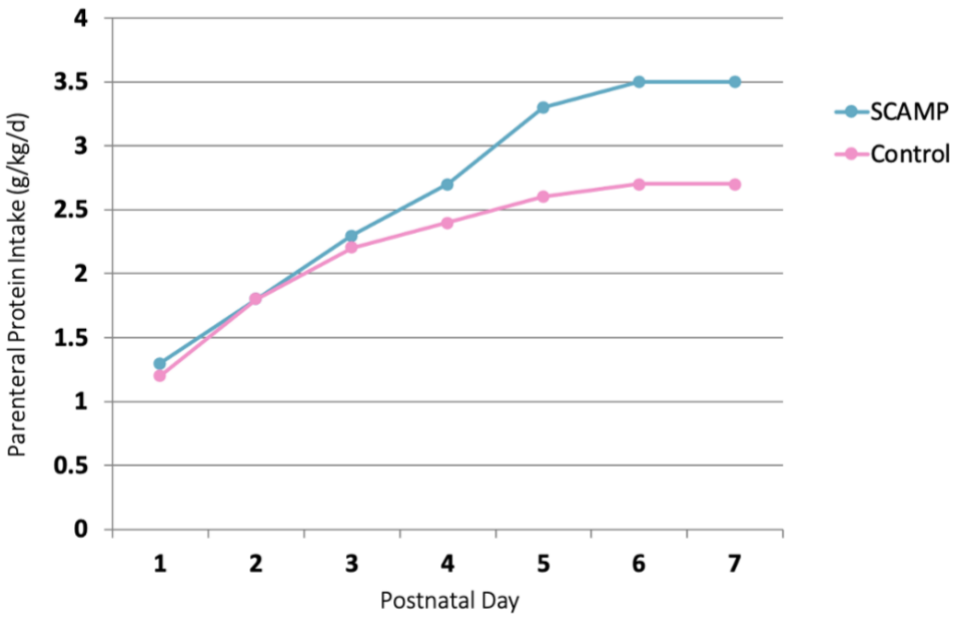


Figure 2.2: Graph showing the incremental increase in parenteral protein intake for SCAMP and control infants

One finding of considerable note is that the plasma AA profile data shows evidence of excess EAA provision in both groups. However, the differences between the groups were not significant, including for leucine, demonstrating no greater EAA overprovision to the infection group. All infants received Vaminolact, a formulation known to result in plasma EAA levels above the recommended reference range (124). Importantly, the re-stratified groups

contained VPN who received the control (2.8g/kg/d) or SCAMP (3.8g/kg/d) maximum protein intake. Still, previous work shows that excess EAA are provided to all infants receiving Vaminolact, regardless of protein intake (124).

Normal physiology indicates that adequate neonatal PN will switch off autophagy compared with a state of starvation (159). We hypothesised that leucine overprovision might impair autophagy, resulting in increased infection. Therefore, it is unsurprising that there are no clinical or statistical differences in infection rates between the groups in a situation where excess EAA are provided for all infants. In the initial infection outcome analysis, Whitby et al. did not report a difference in infection rate between the SCAMP and control groups (170), which is consistent with our negative result. Our data do not support the hypothesis, and as discussed in Chapter 1, there are many other risk factors for neonatal sepsis.

One striking result was that threonine demonstrated great overprovision in all groups, with mean plasma levels more than five times the reference. In neonatal piglets, threonine is required for mucin production and gut function (175). The risk of hyperthreoninemia with neonatal PN has long been recognised (176), and Vaminolact has the highest threonine content of any currently licensed neonatal PN formulations. Limited capacity to metabolise excess threonine has been suggested as the most likely mechanism (124). The safe upper limit of plasma threonine is unknown, but animal studies have raised concerns about the effect of excess threonine on the developing brain (177). Other animal studies indicate that differences in parenteral and enteral threonine metabolism pathways greatly reduce the need for parenteral threonine (178,179). Interestingly, this is the opposite scenario of arginine metabolism, whereby parenteral requirements are increased (163). This highlights how the individual AA requirements of VPN are unique. Threonine is just one example of the major limitations of designing PN formulations using human milk protein or cord blood as a template. Chapman et al. suggested that the threonine requirement for post-surgical parenterally-fed neonates is 22-32% of the threonine content of present commercial PN solutions (180).

Even though the findings were not statistically significant, our data is consistent with existing plasma AA profile data which also suggests PN formulations are imbalanced. Understanding the implications of inadequate PN formulation is particularly important as a complex

relationship between neonatal infections, the inflammatory response and adverse NDOs is slowly emerging. The developing brain, especially the periventricular white matter, is susceptible to cytotoxic and hypoxic injury (81). Infection places infants at an increased risk for abnormal cognitive and motor function (181), something the current PN strategies attempt to avoid.

Interestingly, LOS and NEC both appeared to be associated with aggravation of CEAA deficiency. Glutamine and arginine both had plasma levels below the reference median. The capacity to modulate immune system activity with specific nutrients is termed immunonutrition. The AA most often studied for immunonutrition are arginine and glutamine, examples of deficient AA in PN formulations, or BCAA, including leucine (182).

Glutamine is the most abundant AA in the blood and human breast milk protein (183,184). However, poor stability means glutamine is not included in neonatal PN formulations despite the considerable proportion in breast milk protein. Glutamine has a critical role in several metabolic systems and becomes rapidly depleted in periods of acute illness, trauma and burns (164). In animal models of experimental enterocolitis, glutamine supplementation has been associated with reduced mucosal injury, lower infection rates and increased survival (185). In studies of critically ill adults, results show that glutamine supplementation reduces episodes of sepsis but has little impact on mortality (186,187). Regarding VPN, a meta-analysis of glutamine supplementation in preterm infants suggested that supplemental glutamine does not confer clinically significant benefits (188). Although, the meta-analysis was dominated by a single study (49.8% of infants) which only partly corrected low plasma glutamine (126,189).

Our results showed that plasma concentrations of arginine, another illustration of a deficient CEAA in PN formulations, were lower in infants who developed an infection or NEC. In fact, the SCAMP study reported the lowest published plasma arginine levels in VPN (124), reflecting the low arginine content of Vaminolact. In neonatal piglets, endogenous arginine synthesis occurs from dietary glutamine or proline in the small intestine enterocytes (190). PN is associated with enterocyte atrophy contributing to low levels of intestinal arginine synthesis. Moreover, low plasma glutamine levels may limit endogenous arginine production further (191). Hypoargininemia represents a significant metabolic problem in VPN, first identified 50 years

ago (192). Arginine comprises 14% of tissue protein, meaning the adequate provision of arginine is essential for growth (163). Arginine also plays a central role in several metabolic pathways, including nitric oxide (NO) synthesis and ammonia detoxification by the hepatic urea cycle (193).

Low plasma arginine levels have previously been reported in adults and children with severe sepsis (194,195). Infection is frequent amongst VPN, and elevated cytokines may stimulate the expression of arginase and NO synthase. These enzymes promote arginine catabolism (163), contributing to hypoargininaemia in the infection and/or NEC group. Arginine depletion during infection is a key factor limiting the neonatal ability to mount an adequate immune response (196). Depleted arginine has been shown to affect adaptive immunity through impaired T-cell function (197). One small randomised pilot study investigating arginine supplementation in VPN demonstrated a reduction in NEC incidence (198); others have associated hypoarginemia with NEC (199,200). Furthermore, L-arginine administration may be an effective therapy in infants with persistent pulmonary hypertension of the newborn (PPHN) (201,202).

Recognition of the possible benefits of arginine supplementation is growing and is reflected in recent international guidelines (91). However, AA formulations have not been changed to address these issues in the last 25 years. This is surprising given that, unlike glutamine, arginine is soluble and stable in parenteral solutions. Recruitment is currently underway for PAINT18 (Preterm Arginine INTake 18), an exploratory physiological study investigating the effects of increased arginine (18%) in preterm infants <29 weeks. Evidence from the previous PAINT studies suggests that increasing the proportion of arginine has a 'rebalancing' on the plasma AA profile, increasing arginine levels but simultaneously reducing levels of most essential AA (and leucine is statistically significant). Rebalancing is the first step toward formulating a new generation of AA solutions specifically designed for optimal early postnatal adaptation. The concept of rebalancing PN will be discussed further in Chapter 3, using leucine as an example.

Our study did not find clinically or statistically significant differences between the two groups' total parenteral AA intake or individual AA levels. Even so, there are many other constituents of PN. Hyperglycaemia has previously been associated with increased infection risk in critically ill patients (203). However, PN management protocols and routine biochemical monitoring

effectively limit metabolic complications resulting from increased IV glucose and lipid emulsion infusion rates. In an RCT investigating high-dose, early AA supplementation, recruitment was halted due to a higher occurrence of septicaemia in the intervention group (146). Moltu et al. speculated that hypophosphatemia in the first few postnatal days may have resulted in immunosuppression, contributing to the increased infection rate in the enhanced PN group. The SCAMP study protocol included supplementary electrolyte infusions to allow rapid correction of electrolyte derangement. Even if imbalanced individual AA intakes predispose VPN to infection, the development of neonatal infection is multifactorial and may well be influenced by other PN components.

2.4.1. Limitations

There are some critical limitations of this analysis. Firstly, the original study was not large enough to identify statistical differences in clinical infection rates (170). This is a common theme in neonatal studies, whereby infection-related outcomes are a planned secondary analysis, but the analysis is powered according to the primary outcome. INIS, an international RCT investigating the treatment of neonatal sepsis with intravenous immunoglobulin, required 5,000 infants with proven or suspected sepsis to demonstrate moderate reductions in mortality with adequate power. Recruitment was estimated to take three years, requiring 150 NICUs (204). Therefore, given the clinical significance of the PEPaNIC study findings, it was still important to revisit the existing SCAMP data, despite the small sample size, to explore the possibility of a link between parenteral AA intake and infection before considering designing a larger, expensive study to the same effect.

Furthermore, we suspected the analysis was underpowered, so a retrospective power calculation was performed for the total AA intake analysis. Power was estimated at 0.05, meaning there was only a 5% probability of correctly rejecting the null hypothesis. Generally, an acceptable power is >0.8, meaning there is an 80% or greater chance of correctly detecting a statistically significant result. This analysis was underpowered to detect differences in total AA intake. Therefore it is unclear whether the lack of statistically significant findings is because there was no relationship or because the cohort of 150 infants was insufficient to detect differences in effect size for total intake or plasma AA levels. This prevents any firm conclusions

being drawn from this analysis about the relationship between total AA intake or individual AA concentrations and LOS.

Secondly, multiple statistical tests were run on the dataset given that there were 20 individual AA analysed, amplifying the probability of a false-positive finding. Following Bonferroni correction, the few initially significant results became non-significant, preventing any firm conclusions but still generating future hypotheses about immunonutrition.

Additionally, the infants in the infection group had a significantly lower birth weight than the no-infection group ($z = -3.02$, $p = 0.003$), see Table 2.1. Because the gestational ages were not significantly different between the groups ($z = -1.56$, $p = 0.11$), the infants who developed infections were more likely to be small for gestational age (SGA), a potential confounding variable. Birth weight has long been used to measure immaturity. Therefore, smaller infants may have more underdeveloped GI and metabolic pathways (205), impacting AA metabolism and likely to be reflected in the plasma level. Furthermore, studies have shown that the risk of infection-related mortality and hospital admissions increases with decreasing birth weight (206). Given that this was a retrospective analysis of data generated by a RCT conducted a decade ago and involved analysis of a large number of AA, we chose not to adjust for potential confounders in this analysis. Therefore, the heavy influence of multiple testing and confounders weakens the findings and should be acknowledged when interpreting the clinical importance of results from this study. Our findings suggest that future PN studies should investigate one individual AA as the primary outcome, with the remaining AA as secondary outcomes to minimise the impact of multiple testing.

Plasma AA levels were a secondary outcome measure; the sampling was constrained by routine clinical sampling practice instead of a research protocol. LWH's clinical guidelines recommend collecting samples approximately seven days after maximum AA intake has been achieved and only in infants receiving >50% of nutrition via the parenteral route. Consequently, plasma samples were collected over a wide range of dates, from day 4 to day 25. AA data was missing for some infants; 5 infants died, 11 did not meet the criteria for plasma AA analysis, and three samples were missed. However, the small number of infants with missing plasma data was unlikely to have altered the main study findings. Furthermore, as data was only collected from

the first AA sample obtained, median (IQR) day 9 (8-10), the profile recorded may not represent the AA balance of infants who developed LOS or NEC in the later study period. For example, among the VPN who developed an infection, the median (IQR) day of the first positive blood culture was day 11 (8-14).

Additionally, plasma AA levels are not accepted as an exceptionally reliable measure of assessing AA status because of the impact of metabolism and deposition in organs and tissues. Plasma AA levels represent a delicate balance between AA intake, protein synthesis, degradation, excretion and, for functional AA like leucine, the complex activity of the metabolic pathways (207). The balance is likely to differ between infants with different gestational ages and whether the infant is stable or sick. Sepsis, for example, may increase hepatic protein synthesis to produce acute-phase proteins, increasing proteolysis in peripheral muscle (208). However, a plasma AA profile remains the most clinically accessible measure of the relative proportions of individual AA being metabolised by the body, allowing the identification of potential single AA deficiency or toxicity.

Finally, establishing true rates of neonatal LOS using routine clinical monitoring and laboratory infection markers has critical limitations and is another weakness of this study. A positive BC result remains the gold standard for diagnosis. However, maternal antibiotic therapy can lead to false-negative BC results, and inadequate blood volumes for culture further diminishes the yield (209,210). Therefore, the inability to isolate a microbial pathogen does not necessarily exclude sepsis (211). An unmet area of clinical need and a direction for future research is the development of more efficient diagnostic markers that differentiate infection from other causes of neonatal inflammation and acute deterioration (212).

2.5. Conclusions

In conclusion, there are no significant differences in total AA parenteral intake or plasma EAA levels between infants who develop an infection and/or NEC during the first 28 days of life and infants who do not. However, because the analysis was powered according to the primary outcome, we cannot say with absolute confidence that there were no differences. Overall, the findings together with the hypothesis of this thesis, suggest the PEPaNIC concerns about

parenteral AA provision may not extend to the preterm population. Nevertheless, there continues to be an observable imbalance in EAA and CEAA provision demonstrated by the presence of hypoarginemia and hyperthreoninemia. Reduced plasma concentrations of arginine and glutamine might explain why some infants are predisposed to infection. Correcting the deficiency of these CEAA appears a bigger priority and would have the additional benefit of proportionally reducing plasma EAA levels.

Future studies are required to explore the effectiveness of rebalancing deficient AA as a form of immunonutrition and defining the optimal composition. Further work should calculate the reduction in EAA that would result in plasma levels in the reference range for a preterm infant. The next chapter will investigate the concept of rebalancing for leucine.

Chapter 3: The Relationship Between Parenteral Leucine Intakes and Plasma Leucine Levels in Very Preterm Neonates Dependent on Parenteral Nutrition. A Systematic Review.

3.1. Background

Amino acid (AA) supplementation using current PN formulations may result in overprovision of essential AA (EAA), including leucine. Leucine, a functional AA, is a potent activator of the mTOR pathway. Activation of this pathway increases protein synthesis but inhibits autophagy, a catabolic process involved in the innate immune response. Deficient autophagy following parenteral AA administration is one plausible pathophysiological explanation for poorer clinical outcomes in the early-PN group of the PEPaNIC study. In order to optimise future PN formulations, this chapter details a systematic review of published plasma AA profile data to investigate and quantify the relationship between parenteral leucine intakes and plasma leucine levels in the PN-dependent VPN population.

3.2. Aims

This systematic review addresses the research question, "In VPN, are high intakes of parenteral leucine, compared with low intakes are leucine, associated with appropriate plasma leucine levels?"

- Primary objective - To quantify the relationship between parenteral leucine intake, expressed as percentage leucine content (%) or absolute intake (mg/kg/d), and plasma leucine level (μmol/L).
- Secondary objective - To investigate the effect of study design and different AA chromatography techniques on plasma leucine results. These were studied as potential bias factors that may affect the primary outcome.

3.3. Methodology

The review was designed according to the Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA-P) 2015 (213). The checklist is available in appendix 2. The Cochrane Handbook for Systematic Reviews of Interventions was also used to guide the methodology (214). Ethical approval was not required for this review as it uses existing literature, presenting anonymised patient data.

3.3.1. Search strategy

Four online databases, PubMed, Scopus, Web of Science and Cochrane, were used for this review. Multiple databases were searched to ensure that all the relevant studies were identified and minimise sampling bias. The last searches were performed on the 20th of November 2021.

Individual search strategies were designed for each database according to the PICO formula (Population, Intervention, Comparison, Outcome) breakdown of the main concepts of the research question. The population of interest in this review was PN-dependent VPN. We defined dependency as requiring a minimum of 7 days of PN. This population was selected because VPN are the infant group most vulnerable to malnutrition and most likely to be PN-dependent for an extended period. The intervention and comparator are low versus high parenteral leucine intake expressed as either the percentage leucine content of the AA solution (%) or absolute intake (mg/kg/d). The outcome measured was the plasma leucine concentration (µmol/L). Most published datasets were expected to show overprovision of leucine (plasma concentrations above the proposed reference range mean).

The precision of the search strategy was monitored throughout the search process using a collection of articles reported on by a similarly designed systematic review that investigated the relationship between arginine intake and plasma arginine in the same population (215). 10 of the 12 articles included by Premakumar et al. reported plasma leucine levels in addition to arginine. However, the article by Ng et al. was later excluded because it reported the prescribed AA intake for the PN regimen rather than the actual intake (216). For this review, we chose to include only papers that report actual protein or AA intake because often, due to

practical issues associated with delivering PN, actual intake falls well below the prescribed intake (22,125). The remaining nine articles formed a collection that served as a gold standard for monitoring the accuracy of each search (93,124–126,217–221).

Searches were performed on the four databases using the keywords and synonyms for each PICO concept, as shown in Table 3.1. A basic search performed in PubMed confirmed that the nine gold-standard articles were available. The MeSH (Medical Subject Heading) terms used to index each article were compiled and cross-referenced to capture all relevant articles systematically. MeSH terms were also manually identified from the database thesaurus. The mutual MeSH terms included amino acids, parenteral nutrition and infants. In addition to the MeSH terms, keywords were included to maximise sensitivity by capturing other relevant articles not indexed under the MeSH terms. The MeSH terms and keywords were combined using the OR Boolean operator within each PICO concept. Then, the concepts were combined with the AND Boolean operator to ensure each concept was represented in the final search results. The [tiab] search tag followed each keyword to indicate that the database should search the title and abstract. Preliminary searches generated up to 5,455 results. Following alterations, the PubMed final search strategy outlined in Table 3.2 produced 787 results and included the nine target articles.

POPULATION		INTERVENTION/COMPARATOR: High versus low leucine intake	OUTCOME: Plasma AA level
1 - VPN	2 - PN		
Infant	Parenteral nutrition (PN)	Leucine	Plasma amino acid
Neonate	Total parenteral nutrition (TPN)	Amino acid (AA)	Amino acid level
Newborn	Parenteral nutrition solution	Essential amino acid (EAA)	Amino acid value
Preterm	Parenteral amino acids	Branched-chain amino acid (BCAA)	Amino acid concentration
Premature	Dietary supplement		Aminogram
	Hyperalimentation		
	Intravenous (IV) nutrition		
	Nutritional therapy		

Table 3.1: PICO formula breakdown of concepts

Database searched: **PubMed**	Component of research question and keywords:			
	Population 1	Population 2	Population 3	Intervention/Comparator
Boolean operators	AND	AND	AND	AND
OR	Parenteral nutrition [Mesh]	Infant [Mesh]	Infant* [tiab]	Amino acids [Mesh]
OR	Parenteral nutrition [tiab]	Preterm [tiab]	Neonat*[tiab]	Amino acid* [tiab]
OR		Premature [tiab]		Leucine [tiab]

* Truncation

Table 3.2: Search strategy used on PubMed

A similar search was performed in the Cochrane Library, which yielded eight articles from the initial collection; the remaining article by Ogata et al. (221) was unavailable on the Cochrane database. The search strategy was adapted to include and exclude certain search terms to ensure the eight available articles were generated. For example, the search strategy did not generate five of the gold-standard articles without including the MeSH term "parenteral nutrition solution". The final search strategy is available in Table 3.3.

Database searched: **Cochrane**	Component of research question and keywords:		
	Population 1	Population 2	Intervention/Comparator
Boolean operators	AND	AND	AND
OR	Parenteral nutrition [MeSH]	Infant [MeSH]	Amino acids [MeSH]
OR	Parenteral Nutrition Solutions [MeSH]	Infant*	Leucine
OR	Dietary Supplements [MeSH]	Premature	Amino acid*
OR	Parenteral nutrition	Preterm	Essential amino acid*
OR	Intravenous nutrition		
OR	Hyperalimentation		
OR	Parenteral amino acid*		

* = Truncation

Table 3.3: Search strategy used on Cochrane

A similar approach was used for the searches conducted using Scopus and Web of Science. Each search included a broad set of search terms and was adapted to include the database's specific operators and field tags. The search was stopped when the addition of new terms yielded no new relevant results or when the removal of terms resulted in relevant articles being excluded. The final search strategies are available in Table 3.4 and Table 3.5. Finally, a manual search was conducted of the bibliographies of relevant articles, which generated no additional results.

Database searched: **SCOPUS**	Component of research question and keywords:			
	Population 1 [a]	Population 2 [a]	Intervention/ Comparator [a]	Outcome
Boolean operators	AND	AND	AND	AND
OR	Parenteral nutrition	Infant*	Leucine	Plasma amino acid*
OR	Total parenteral nutrition	Preterm	Amino acid*	Plasma amino acid level*
OR	Parenteral nutrition solution*	Premature	Essential amino acid*	Plasma amino acid value*
OR	Parenteral amino acid*			Plasma amino acid concentration*
OR	PN			Aminogram*
OR	TPN			Amino acid level*
OR	Dietary supplement*			Amino acid concentration*
OR	Hyperalimentation			
OR	Intravenous nutrition			
OR	IV nutrition			
OR	Nutritional therapy			

[a] The TITLE-ABS-KEY field code was used before this concept

Table 3.4: Search strategy used on Scopus

Database searched: **Web of Science**	Component of research question and keywords:			
	Population 1	Population 2	Intervention/ comparator	Outcome
Boolean operators	AND	AND	AND	AND
OR	Parenteral nutrition	Infant	Leucine	Plasma amino acid*
OR	Total parenteral nutrition	Neonate	Amino acid*	Plasma amino acid level*
OR	Parenteral nutrition solution*	Preterm	Essential amino acid*	Plasma amino acid concentration*
OR	PN	Premature	Branched-chain amino acid*	Plasma amino acid value*
OR	TPN	Newborn		Aminogram
OR	Dietary supplement*			Amino acid level
OR	Intravenous feed*			Amino acid value
OR	IV feed*			
OR	Hyper$alimentation			
OR	Intravenous hyperalimentation			
OR	IV hyperalimentation			
OR	Parenteral hyper$alimentation			
OR	Intravenous nutrition			
OR	IV nutrition			
OR	Amino acid solution			

Table 3.5: Search strategy used on Web of Science

3.3.2. Inclusion and exclusion criteria

No restriction was applied to the search regarding publication date; this was important because the data from older studies benefited this review as they report on AA solutions that are no longer used. Older AA solutions had a different composition than contemporary solutions, increasing the leucine intake data range. All study designs were eligible for inclusion, except for review articles, to ensure that data was not missed from observational or non-randomised studies. Studies of infants born with gestational age ≤32 weeks (VPN) were eligible. In two articles which went on to be included, the mean gestation of the sample was less than 32 weeks, but the range extended above 32 weeks (222,223). In cases such as these, provided the upper limit was less than 34 weeks, articles were still included in the review as most infants studied were classed as very preterm. Both studies including infants >32 weeks were small (15 and 20 VPN), therefore only a small group infants were outside the desired population.

The following additional study characteristics were required for inclusion in this review: (1) Infants studied within the neonatal period (the first 28 days of life); (2) Neonates received PN as the primary nutrition source for >7 days; (3) Protein or AA intake reported along with the name of the AA solution used. Only articles that reported plasma AA levels after three days (72 hours) of study PN were included to ensure target AA intake was established before the sample was collected.

During the study selection process, an inclusion criterion was added to specify that blood samples for plasma AA analysis should be collected as heparinised whole blood. Any studies that analysed dried blood spots (DBS), small samples of whole blood blotted onto absorbent paper, were excluded because the validity of DBS for monitoring leucine level is an ongoing debate due to concerns over the separation of BCAA (224). Most studies comparing the two blood sampling methods focus on monitoring phenylalanine and tyrosine concentrations in patients with Phenylketonuria (PKU) and Tyrosinemia type 1 (TT1), two examples of inherited metabolic diseases identified by the newborn screening DBS. A DBS-plasma correction factor is often used for DBS measurement to compare the two types of blood samples. Each laboratory determines its own correction factor dependent on filter card type, extraction and calibration protocols using the heparinised plasma values as the gold standard (225). For this

reason, only plasma samples collected from heparinised whole blood were included in this review to prevent differences arising between sampling methods and allow a direct comparison of plasma leucine levels.

The inclusion and exclusion criteria are summarised according to the PICO formula in Table 3.6.

	INCLUSION CRITERIA	EXCLUSION CRITERIA
POPULATION 1: PN dependent	- Neonates receiving PN as the primary source of nutrition for ≥7 days	- Neonates receiving PN for <7 days - Neonates receiving significant enteral feeds (>50%) by the time the sample was collected
POPULATION 2: Very preterm neonates	- Human subjects - Newborn babies within the first 28 days of life (neonates) - Babies born with gestational age ≤32 weeks (VPN)	- Animal subjects - Infants (>28 days of age), children and adults - Neonates born >32 weeks' gestation
INTERVENTION AND COMPARATOR: Leucine intake	- Received a named AA solution with a known leucine content - Actual protein/AA intake reported	- Composition of AA solution unavailable in the article or online resources - Prescribed protein/AA intake reported instead of actual intake
OUTCOME: Plasma Leucine levels	- Plasma AA concentrations reported, including plasma leucine - Plasma levels reported from day 4 of PN onward (>72 hours) - Analysis of plasma obtained from a heparinised whole-blood sample	- Plasma concentrations are available for other AA but plasma leucine concentration unreported - Analysis of a dried blood spot (DBS) - Plasma levels reported before day 4 (<72 hours) of PN
TYPE OF STUDY	- Randomised control trials or observational studies (including cohort, case-control and cross-sectional studies) - Studies published in any language	- Systematic reviews, case studies or other secondary data

Table 3.6: Inclusion and exclusion criteria based on PICO strategy

3.3.3. Data extraction

From each included article, pertinent information was extracted from each paper so that the research question could be answered and the quality of the paper assessed. The information obtained included the study design and objectives, study population, details on the PN solution used, PN protocol and the plasma leucine level, including the day of sample and method of analysis. When data was unavailable in an article, the missing information was searched for in other reports of the same trial. Some articles reported the AA solution used but not the composition of the solution; in these cases, the composition of the solution was obtained from reliable online resources such as the manufacturer's summary of product characteristics (SmPC) document. The absolute leucine intake (mg/kg/d) was calculated by multiplying the actual protein intake (g/kg/d) by the percentage of leucine in the PN solution.

The data was collected using the predesigned proforma shown in Figure 3.1 to ensure that the data collection process was consistent across the different papers. The draft proforma was first piloted on a small group of papers and then modified to include and exclude certain fields before finalising the data collection form. Once extracted, the data was recorded onto a Microsoft Excel spreadsheet and finalised between KD and supervisor Colin Morgan (CM). The complete data extraction spreadsheet is attached as Appendix 3.

DATA EXTRACTION FORM

Reviewer: Date form completed:

DETAILS OF THE STUDY
- Study ID:
- Title:
- Authors:
- Publication year:
- Publication source:
- Funding source:

OVERALL STUDY BACKGROUND
- Study design:
- Study period:
- Study setting/location:
- Ethics:
- Consent:
- Randomisation:
- Primary outcome(s) and measurement methodology:
- Secondary outcomes(s) and measurement methodology:

POPULATION
- Sample size:
- Inclusion criteria:
- Exclusion criteria:
- Gestational age (weeks):
- Birth weight (g):
- Gender:
- Co-existing conditions:
- Severity of illness:
- Other treatment(s) received:

INTERVENTION
- Route of PN:
- Age at initiation of PN:
- Duration of PN:
- AA intake:
- Enteral supply:

Group A / B / C details:
- Number randomised to the group:
- PN solution used:
- Percentage leucine:
- Description of PN regimen:

PLASMA AA RESULTS
- Number of participants with plasma AA data:
- Reported leucine intake:
- Group A/B/C -
- Reported plasma leucine level:
- Group A/B/C -
- Day of plasma sample:
- Sampling protocol:
- AA analytical assay method:
- Reference range and source:

Figure 3.1: Data extraction tool

3.3.4. Statistical analysis

Statistical tests were performed using Statistical Package for the Social Sciences (SPSS) 22 [IBM Corp. Released 2020. IBM SPSS Statistics for Macintosh, Version 27.0. Armonk, NY: IBM Corp]. Correlation and regression analysis was conducted to quantify the relationship between parenteral leucine intake and plasma leucine level and to understand the strength of these relationships. Percentage leucine content (%) and absolute intake (mg/kg/d) were plotted against plasma leucine level (µmol/L) on scatterplots to give two dose-concentration graphs. Subgroup analysis was also done based on the chromatography method, ion-exchange chromatography (IEC) versus high-performance liquid chromatography (HPLC), used for the plasma AA analysis. A p-value of ≤0.05 will be considered statistically significant.

The data collected demonstrated high variability between treatment groups within each individual study and between the 12 studies included in this analysis. The heterogeneity was most likely a result of the range of clinical settings and time periods, meaning that different nutrition guidelines and hospital policies regarding AA and energy intakes influenced each study's protocol. In addition, there were different study designs, multiple PN formulations studied and different AA analysis techniques used. As it was expected that the studies yielded by the literature search would be too heterogeneous, a meta-analysis was not planned. This is in agreement with findings from the previous systematic review by Premakumar et al. which used a similar methodology (215).

3.3.5. Selection of a reference range

There is controversy surrounding the optimum mean plasma leucine level for the VPN population. For this review, 112 µmol/L was used as the desirable mean plasma leucine level. 112 µmol/L was selected based on plasma leucine levels (mean ± SD) from a range of neonatal populations identified in published studies (Table 3.7). A different reference range was used in Chapter 2, in keeping with other analyses of the SCAMP data. However, that reference range was not included in Table 3.9 because it included infants up to 6 months of age, and for this analysis we were interested in solely neonates (infants within the first 28 day of life). Reference plasma levels range from the lowest value of 53.2 µmol/L to the highest value of 171 µmol/L, indicating an apparent lack of consensus on acceptable plasma levels. 112 µmol/L is a

pragmatic target because it is the midpoint and close to the reference range for a term, breastfed infant.

Population	Reference range (μmol/L)	Reference
Healthy, term, breastfed infants with postnatal age between 28 and 32 days	111.3 ± 27.3 Target range of 53.2-169.4	(226)
Healthy, term, breastfed infants with postnatal age of 11 days	119 ± 25 Target range 86 - 171	(227)
Preterm, human-milk-fed infants	87 ± 14	(228)
Extremely low-birthweight (ELBW) infants	114.7 ± 51.4	(126)
Cord blood from neonates of 29 weeks' gestational age	153 ± 36	(229)

Table 3.7: Plasma leucine levels of five comparative neonatal groups

3.3.6. Quality appraisal

Each included study was assessed using the quality assessment tool (QAT) available in Appendix 4. The tool was modelled on the one used by Premakumar et al. (215) and was designed to compare the quality of the included articles and evaluate the risk of bias at a study level. Two existing tools were used to design the QAT, ROBIS Tool (230) and QUADAS-2 Tool (231), to assess the risk of bias in systematic reviews. A number of articles were also used to tailor questions to screen for good reporting of neonatal outcomes (232,233). Finally, the articles outlined in Table 1.4 were re-evaluated to determine which nutrition details were commonly reported in published neonatal nutrition studies. The tool was piloted using the inclusion articles then finalised with supervisors CM and MT.

The tool comprised 43 questions and was broken down into six sections reporting different aspects of the study design. A point was given for every relevant question in the tool if the study reported the requested detail. The final score was reported as a percentage to quantify the quality of the study, allowing direct comparison between the 12 articles. Additionally, bias was assessed across the whole review using the GRADE (Grading of Recommendations, Assessment, Development and Evaluations) framework.

3.4. Results

3.4.1. Study selection

A combined total of 1,472 papers, including duplicates, were generated by the four database searches. No additional articles were identified from the bibliographies of relevant papers or other sources. For the purpose of the thesis, study selection was performed by a single reviewer, Keziah Davies (KD). Due to the time constraints of an MPhil, it was not possible for conventional double-screening. However, the work presented in this Chapter will hopefully be developed into a future publication. Therefore, the intention is to recruit a second reviewer who will independently screen articles, increasing the reliability of the results by minimising the non-detection of relevant articles which confers a potential risk of bias.

First, the results were filtered based on the availability of the full text in the English language. For the PubMed search results, an additional search filter of 'human' was used to eliminate 46 animal studies. The search results were screened by title, abstract and full text, as illustrated by the PRISMA flow diagram (Figure 3.2), which displays the number of papers included and excluded at each stage. Articles with titles that were unrelated to the research question were excluded. Next, the abstracts were read to determine if the article remained relevant. Finally, the full text of each article was retrieved and read to determine whether the study met the inclusion and exclusion criteria.

Two additional articles that met the predefined criteria were later excluded because they did not report plasma leucine concentration according to the AA solution. The article by Burgess et al. reported plasma according to insulin-treated and non-insulin-treated groups (234). A second article by Clark et al. (102) reported plasma leucine based on two groups receiving different doses of AA intake. However, within those separate dose groups, the neonates had received two different AA solutions, and the results were not separated based on the type of AA solution used. For both articles, it was impossible to plot the dose-concentration graphs between the plasma levels and leucine intakes from a particular AA solution.

At this stage of the screening process, there were 9 eligible articles from Cochrane, 11 from PubMed, 11 from Scopus and 5 from Web of Science. Once duplicates were removed, 12 articles that fulfilled all criteria were included in the review.

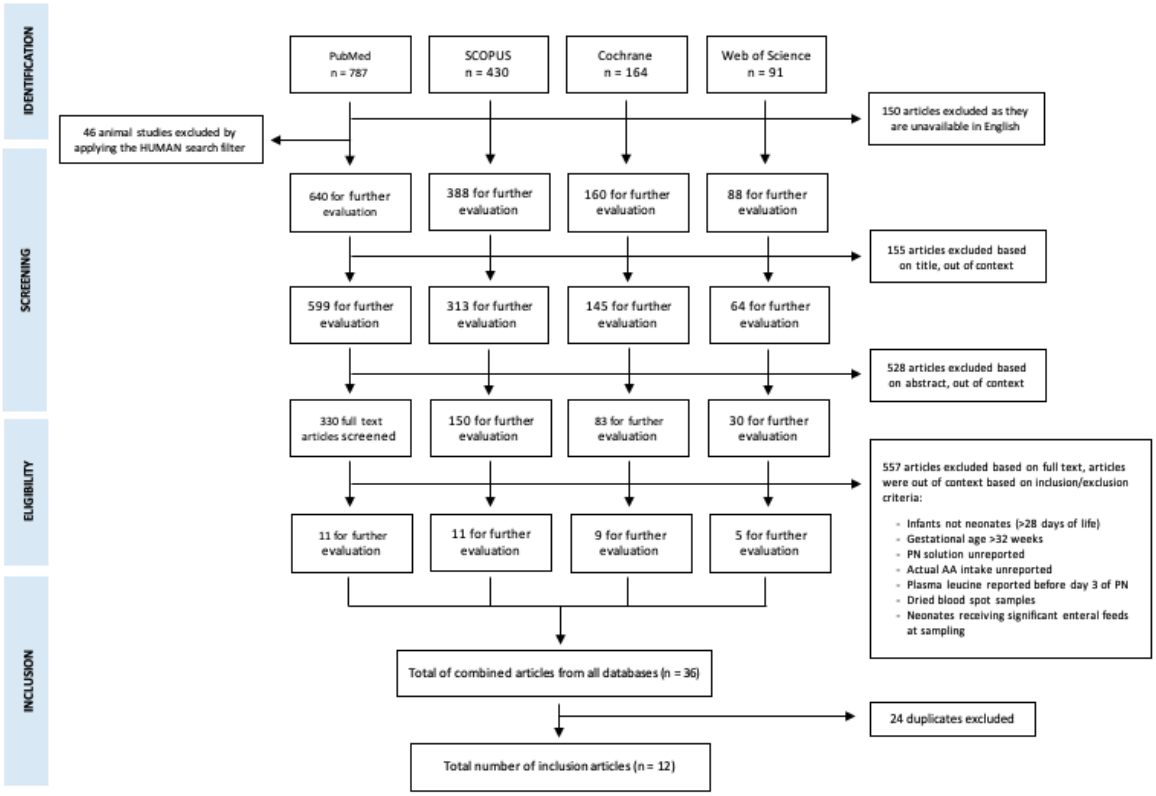


Figure 3.2: PRISMA diagram outlining the article screening process for the systematic review

3.4.2. Study characteristics

Out of the 12 final articles, there were nine randomised trials (RCT), one non-randomised control trial, one cohort study and a final study with a crossover design. The studies were published between 1983 and 2017.

A total of 650 VPNs are included in this systematic review. Sample sizes for individual studies ranged from 15 to 141 VPNs. Both male and female infants were studied, and infants had a minimum and maximum gestational age of 23.7 and 33.3 weeks, and a mean of 27.9 weeks. The AA formulations used were Aminosyn 10%, Aminovenos 10%, Neopham, Primene 10%, TrophAmine 10%, Travasol 10% blends B and blend C, Vamin 9 glucose, Vamin Infant and Vaminolact. The data extracted from each paper is summarised in Table 3.8.

Author (Year)	Gestational Age (Weeks)	Amino Acid Solution	Leucine content (%)	Leucine Intake (mg/kg/d)	Plasma Leucine Level (µmol/L)	Number of AA profiles	Day of Sample	Analysis Method
Blanco et al. (2011) (93)	1a: 25.7 ± 2 1b: 26.3 ± 2	1: Aminosyn PF 10%	1: 11.9	1a: 440.3 1b: 345.1	[a] 1a: 202.6 (136.8 - 277.5) [a] 1b: 192.5 (156.0 - 235.3)	55	Day 7 of PN	HPLC
Bulbul et al. (2012) (217)	2a: 29.4 ± 1.8 2b: 29.1 ± 1.1	2: Primene 10%	2: 9.9	2a: 356.4 2b: 356.4	2a: 100.0 ± 28.4 2b: 109.0 ± 72.4	44	7th postnatal day	HPLC
* Chessex et al. (1985) (218)	3: 28 ± 1	3a: Started on Travasol 10% blend B 3b: Started on Vamin 7%	3a: 6.2 3a: 7.5	3a: 167.4 3b: 202.5	3a: 71 ± 18 3b: 106 ± 23	15	Day 6 of each period of PN	IEC
Kalhan et al. (2005) (219)	4a: 26.7 ± 1.6 4b: 27.7 ± 2.0	4a: TrophAmine 10% + glutamine 4b: TrophAmine 10%	4a: 11.2 4b: 14.0	4a: 358.4 4b: 350.0	4a: 109.1 ± 21.4 4b: 124.6 ± 22.17	20	After 3-5 days of PN	HPLC
Mayes et al. (2014) (125)	5a: 26.0 ± 1.5 5b: 26.2 ± 1.5	5: Primene 10%	5: 9.9	5a: 277.2 5b: 217.8	5a: 130 (104 - 156) 5b: 111 (86-129)	118	Day 8 - 10 of life	IEC
Mitton et al. (1992) (220)	6a: 29 ± 3 6b: 29 ± 2	6a: Vamin 9 glucose 6b: Vamin Infant	6a: 7.5 6b: 10.7	6a: 240.0 6b: 345.6	[b] 6a: 125 (51 -204) [b] 6b: 135 (88 – 195)	29	Day 5 of PN	IEC
Morgan and Burgess (2015) (124)	7a: 26.8 ± 1.3 7b: 26.6 ± 1.4	7: Vaminolact 6.5%	7: 10.7	7a: 382 ± 113 7b: 318 ± 50	7a: 156 (131 - 169) 7b: 136 (122 - 156)	126	Day 9 (8 - 10) of life	IEC
Ogata et al. (1983) (221)	8a: 28.0 ± 1.6 8b: 28.2 ± 0.7	8a: Neopham 8b: Aminosyn	8a: 10.8 8b: 9.5	8a: 276.48 8b: 255.55	8a: 111 ± 39 8b: 91 ± 20	17	Day 7 of infusion	Not reported
* Pineault et al. (1986) (235)	9: 27 ± 0.5	9: Travasol 10% blend C	9: 7.3	9: 190.53	[c] 9: 68 ± 4	10	After 4.6 ± 0.3 days of infusion	IEC
Poindexter et al. (2003) (126)	10a: 26.2 ± 2.0 10b: 26.3 ± 1.8	10a: TrophAmine 10% + glutamine 10b: TrophAmine 10%	10a: 11.2 10b: 14.0	10a: 268.8 10b: 306.6	10a: 100 (80 - 126) 10b: 118 (89 - 142)	141	After 10 days of PN	IEC
* Thornton and Griffin (1991) (222)	11: 29.7 ± 3.6 [d]	11: Vaminolact 6.5%	11: 10.7	11: 246.1	11: 166 ± 56	15	After 3 days of 2.5g/kg/d intake	IEC
Van Goudoever et al. (1994) (223)	12a: 31 ± 2 [d] 12b: 30 ± 2 12c: 30 ± 2	12a: Aminovenos-N-pad 10% 12b: Vaminolact 6.5% 12c: Primene 10%	12a: 10.3 12b: 10.7 12c: 9.9	12a: 236.9 12b: 224.7 12c: 217.8	12a: 86 ± 19 12b: 102 ± 23 12c: 119 ± 19	20	Day 7 of life	IEC

Table 3.8: Summary of data extraction

Results are expressed as mean ± standard deviation (SD) or median (Q_1 –Q_3) unless indicated otherwise, [a] Median (10^{th} to 90^{th} percentile range), [b] Mean (95% confidence intervals), [c] Mean ± SEM
[d] Papers with a mean gestation ≤32 weeks but an upper standard deviation extending above 32 weeks
* Non-RCT study design

3.4.3. Evaluation of the dose-response relationship

Once collected, the data was plotted on two scatter graphs whereby the y-axis represented plasma leucine concentration (µmol/L), and the x-axis was labelled as either percentage leucine content (%) or absolute leucine intake (mg/kg/d). The points were coded by colour and shape according to the AA solution used (Primene, Vaminolact, TrophAmine or Other). Each point (n = 22) was labelled by article number (1 to 12) and by treatment group (a, b or c) as outlined in Table 3.8. The scatterplots are shown in Figure 3.3.

There was a moderate positive correlation between the percentage leucine content (%) of the AA solution and the plasma leucine level (µmol/L), shown in Figure 3.3a, which was confirmed by a Pearson's correlation coefficient of r = 0.488, n =23, p = <0.01. The correlation between absolute leucine intake (mg/kg/d) and plasma leucine, shown by Figure 3.3b, was a stronger positive correlation (r = 0.640, n = 23, p = <0.001).

A simple linear regression model was used to predict plasma leucine concentration based on percentage leucine content. A significant regression equation (P <0.05) was found with an R^2 value of 0.238, indicating 24% of variance in plasma leucine can be explained by a model containing only percentage leucine. The predicted plasma leucine level equals 30.73 + 8.73(percentage leucine content). For every 1% increase in percentage leucine content, plasma leucine is expected to increase by 8.73 µmol/L.

The regression analysis was repeated to predict plasma leucine concentration from absolute leucine intake. Again, a significant regression equation (p = 0.001) was found, with a higher R^2 value of 0.409. The regression equation predicted plasma leucine level equals 0.3(absolute leucine intake) + 33.83. Therefore, plasma leucine increased by 0.3 µmol/L for every 1 mg/kg/d increase in absolute leucine intake.

Multiple linear regression was calculated to predict plasma leucine level based both on percentage content and absolute intake. The predicted plasma leucine is equal to 2.4(percentage leucine content) + 0.3(absolute leucine intake) + 21.1. The multiple regression equation was statistically significant (p <0.05), with an R^2 value of 0.361. However, only

absolute leucine intake significantly predicts plasma leucine level in this model ($p < 0.05$). This reinforces the previous finding that absolute leucine intake has a stronger correlation with plasma leucine intake.

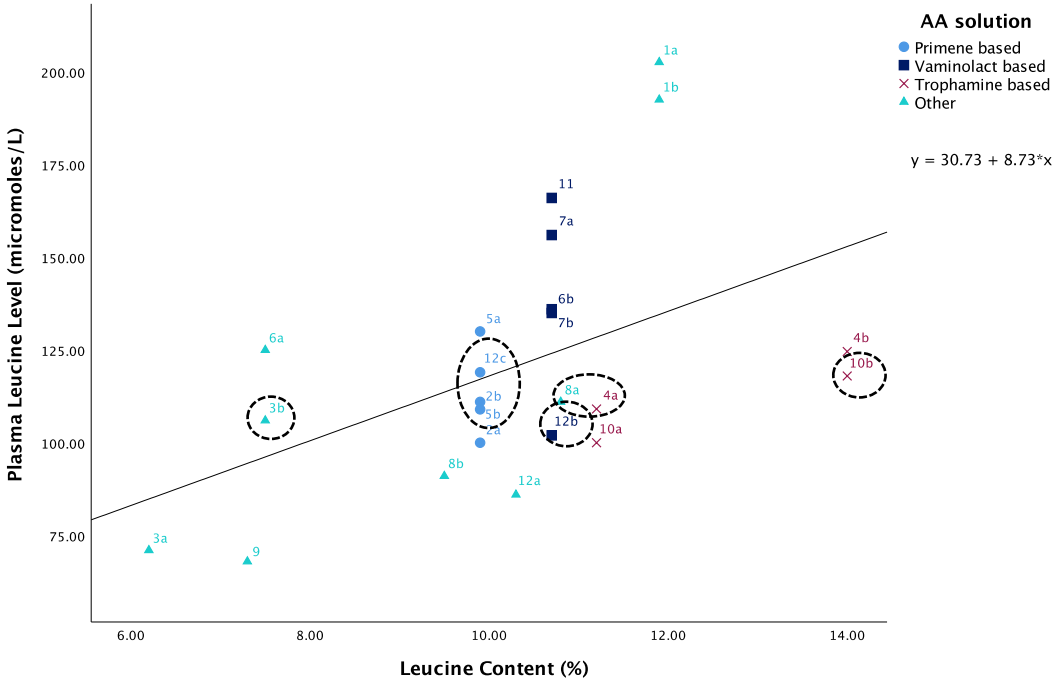


Figure 3.3a: Dose concentration graph of percentage leucine content with plasma leucine level

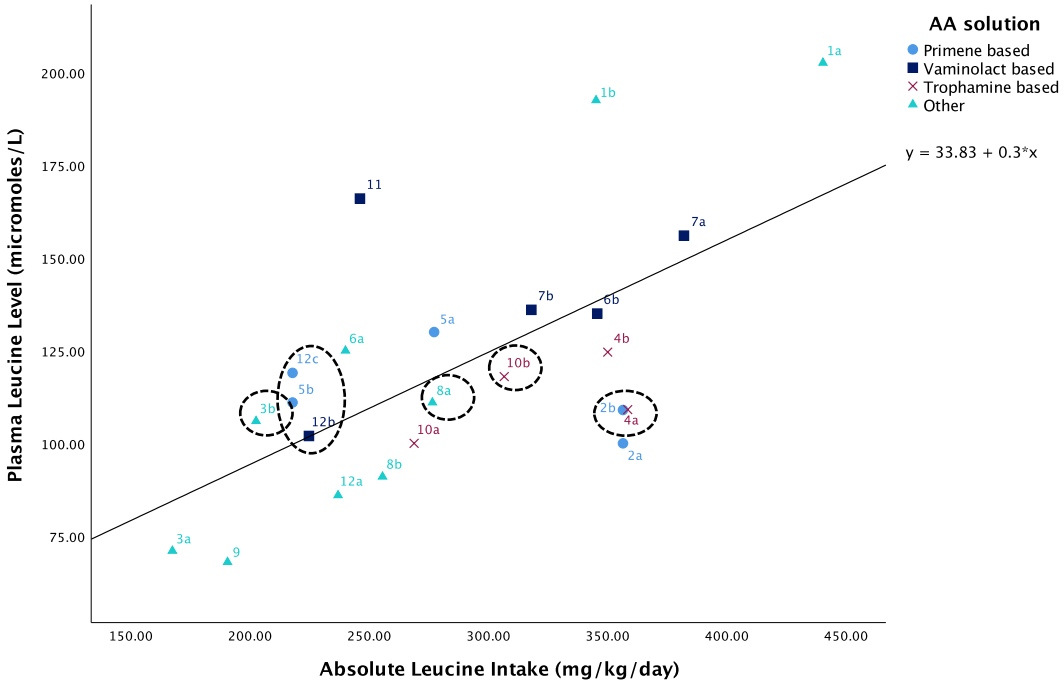


Figure 3.3b: Dose concentration graph of absolute leucine intake with plasma leucine level

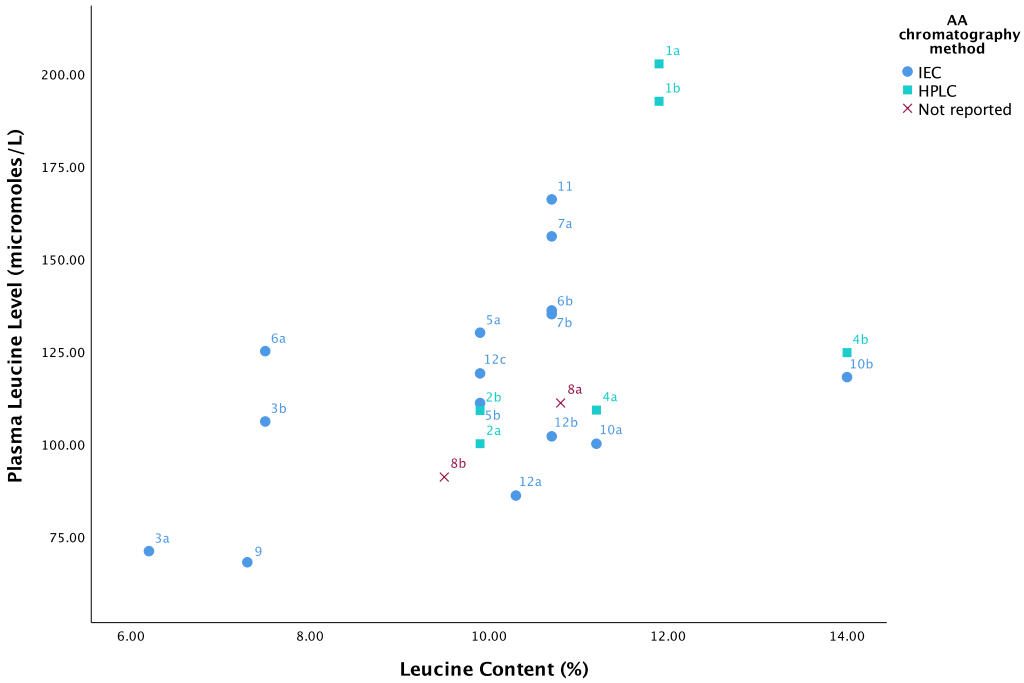
Figure 3.3: Dose concentration graphs of leucine content, expressed as a percentage content (3.3a) or absolute intake (3.3b), with plasma leucine level. Refer to Table 3.8 to identify the article and treatment group for each data point. Circled points are explained in the discussion.

3.4.4. Study of bias factors

Further data analysis was performed to ascertain whether study design features influenced the outcome. Analysis of study design (RCT versus non-RCT) was performed and found no significant difference and therefore is not reported.

Another factor studied was the method of chromatography used for plasma AA analysis, which appeared to have a small effect on the dose-concentration relationship. Eight articles used IEC, and three used HPLC. The article by Ogata et al. did not report the chromatography method used and was excluded from this analysis (221). Figure 3.4 shows the data points on the graph coded according to the AA chromatography method. Overall, the data points analysed by HPLC (n = 15) had higher plasma leucine levels with a mean of 139.6 µmol/L, Standard Error of the Mean (SEM) = 18.6. Whereas groups analysed by IEC (n = 6) had a mean of 115.3 µmol/L, SEM = 7.2. However, it was not appropriate to statistically compare the two methods as they were performed using different equipment and on samples collected from different infants receiving different nutrition regimens. Therefore, further statistical analysis would not be valid.

Dose concentration relationship graph of percentage leucine content with plasma leucine level



Dose concentration relationship graph of absolute leucine intake with plasma leucine level

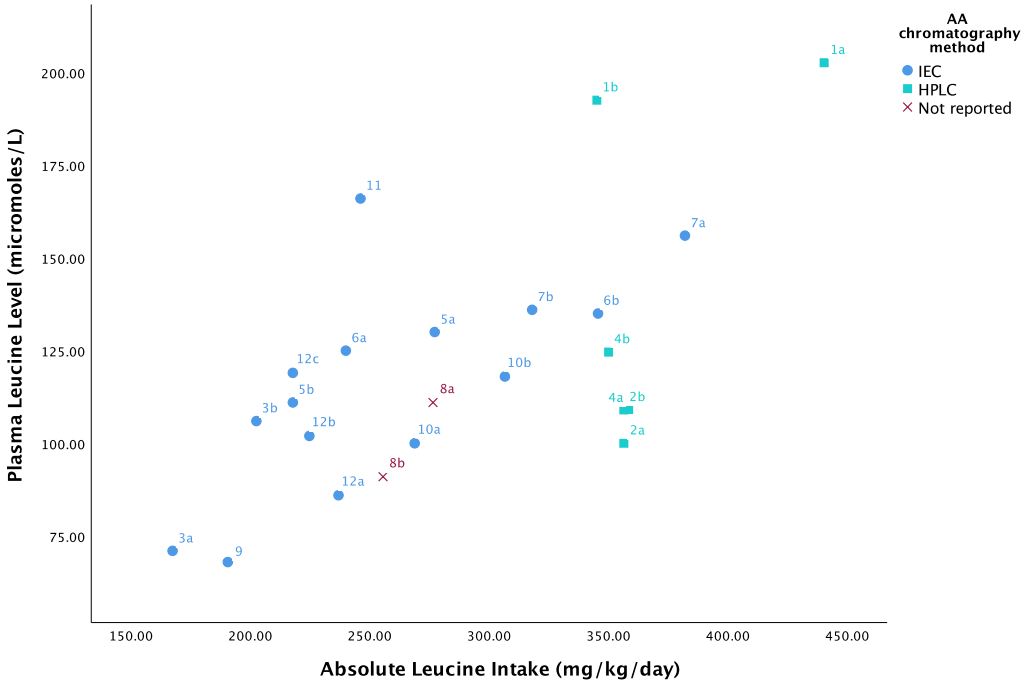


Figure 3.4: Dose concentration graphs coded according to the chromatography method used for AA analysis. Refer to Table 3.8 to identify the article and treatment group for each data point.

3.4.5. Quality appraisal

A customised quality assessment tool (QAT), designed for this systematic review, enabled the comparison of quality between differently designed studies. The percentage score ranged from 61.4% to 91.1% for each paper, with a mean percentage score of 76.0%. The complete quality assessment findings are provided in Appendix 5. A scatterplot between the study size and percentage quality showed no significant relationship, confirmed by a Pearson's correlation coefficient of 0.182 ($p = 0.571$).

A summary of the risk of bias (RoB) assessment based on the 'traffic light' colour code (red for high risk, yellow for uncertain and green for low risk) is shown in Table 3.9 (236,237). The tool assesses the quality of evidence presented by the individual studies included in the systematic review. Even though it was designed for randomised trials, some bias domains apply to other study designs. Therefore, the tool was used for all the papers included in the systematic review.

The GRADE assessment tool was also used to evaluate the certainty of the body of evidence presented concerning the primary outcome of the systematic review (238). Overall, the certainty would be categorised as low based on an assessment of the weaknesses in the five GRADE domains: risk of bias, imprecision, inconsistency, indirectness of evidence and publication bias.

	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessors (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other sources of bias
Blanco et al. (2011)	?	+	+	+	+	+	+
Bulbul et al. (2012)	+	+	+	+	+	+	+
Chessex et al. (1985)	?	?	?	?	+	+	+
Kalhan et al. (2005)	?	?	?	?	+	+	+
Mayes et al. (2014)	+	+	+	+	+	+	+
Mitton et al. (1993)	?	?	?	?	+	+	+
Morgan and Burgess (2017)	+	+	+	+	+	+	+
Ogata et al. (1983)	?	+	+	+	+	+	+
Pineault et al. (1986)	n/a	n/a	n/a	n/a	+	+	-
Poindexter et al. (2003)	+	+	+	+	+	+	+
Thornton and Griffin (1991)	n/a	n/a	?	?	+	+	-
Van Goudoever et al. (1994)	?	?	?	?	+	+	+

Table 3.9: Risk of bias assessment using the Cochrane risk of bias tool. Red indicates high risk, yellow for uncertain and green for low risk.

3.5. Discussion

3.5.1. Summary of main results

This systematic review summarises the relationship between parenteral leucine intake, expressed as percentage content (%) or absolute amount (mg/kg/d), and plasma leucine level (µmol/L) in the PN-dependant VPN population. Although the included studies did not intend to investigate the link between leucine intake and plasma leucine level as a primary outcome, sufficient information could be extracted from each article to quantify the relationship between the two variables. This relationship has been clearly defined using intake and plasma AA data from 12 previous studies, which collectively studied 650 VPN. Moreover, this approach enables the estimation of a minimum leucine intake required to achieve an acceptable plasma leucine range.

3.5.2. Evaluation of the dose-response relationship

The correlation and regression analysis indicated that both percentage leucine content and absolute leucine intake predict plasma leucine level. It was essential to consider both aspects since there was no clear prior understanding of which was a stronger predictor of plasma level. It was also important to ensure that both AA composition (quality) and quantity effects were considered. The scatterplots in Figure 3.3 show a linear relationship for both independent variables. These dose-response relationships suggest that modifying the absolute intake of leucine and the proportion of leucine will change plasma levels.

The higher R^2 value for the simple regression equation and the statistical significance of absolute leucine intake in the stepwise multiple regression model indicates it was the stronger predictor of outcome. This effect can be illustrated using data points 7a and 7b as both study groups received Vaminolact, a solution which contains 10.7% leucine. However, infants in group 7a received the SCAMP regimen and a higher protein intake of 3.66 g/kg/d compared to the control infants (7b), who received 3.02 g/kg/d. The plasma leucine levels were lower for point 7b, reinforcing that a higher absolute leucine intake, determined by actual AA intake, results in a higher plasma level.

On the other hand, examining the points with an absolute leucine intake of approximately 350 ± 10 mg/kg/d (1b, 2a, 2b, 4a, 4b and 6b), the different studies have used various AA solutions (Aminosyn PF 10%, Primene 10%, TrophAmine 10% and Vamin, a solution identical to Vaminolact). Each solution contains a different percentage content of leucine ranging from 9.9% to 14%, resulting in a range of plasma levels (100 to 342.4 µmol/L). This suggests that even though percentage content was not significant in the multiple regression model, changing the percentage of leucine in an AA solution would still affect the plasma leucine levels. Therefore, both aspects of intake should be considered in future formulation design studies as both can impact the plasma AA outcome.

For this review, 112 µmol/L will be used for a worked example, to demonstrate how the regression equations could be used to predict leucine intake for a desired plasma leucine level. 112 µmol/L was selected based of plasma leucine levels collected from a range of neonatal populations identified in published studies (Table 3.7). Eight data points (2b, 3b, 4a, 5b, 8a, 10b, 12b and 12c) achieved a plasma leucine level within 10 µmol/L of the target concentration of 112 µmol/L. These points have been circled in Figure 3.3 to illustrate this finding. For this collection of study groups, the percentage leucine content ranged from 9.9% to 14.0%, and the absolute leucine intake ranged between 202.5 and 358.4 mg/kg/d.

The linear regression equations and target concentration of 112 µmol/L can estimate the minimum absolute leucine intake and percentage content required to achieve an acceptable plasma leucine level. The intakes proposed here are based on speculation and assumptions due to the difficulties quantifying metabolic demand for individual AA. Defining these demands in a neonatal population is even more complex because metabolic requirements can differ based on gestational age at birth. Furthermore, VPN are a vulnerable and sick population with increased metabolic needs, which are a higher priority than protein synthesis. Consequently, the AA supply meets acute metabolic needs before being used for protein synthesis. Again, this is difficult to quantify, and it is unclear how the plasma AA concentration reflects the balance between intake, protein synthesis and utilisation in metabolic processes.

According to the simple regression equation for percentage leucine content, the proportion of leucine required for a plasma level of 112 µmol/L was estimated to be 9.3%. Unsurprisingly,

this was lower than the leucine percentage in all the modern solutions studied, including Primene, Vaminolact and TrophAmine. This finding reiterates concerns that PN is imbalanced, delivering excess EAA and insufficient CEAA. The same imbalance is found in the three most widely used neonatal AA solutions (Primene (125), Vaminolact (124) and TrophAmine (126).

We have previously shown how a systematic review of published plasma AA data can calculate the optimal neonatal PN content of a deficient AA, arginine (215). Adapting the methodology used by Premakumar et al. demonstrated that our approach also works for an AA provided in excess. While this approach has limitations, particularly the heterogenicity between studies, it offers a systematic approach to rebalancing parenteral AA formulations using already published data. Our approach could be used to optimise the remaining AA, although there remain solubility and stability issues that should be tackled first.

Our data provide a methodology which may enable the concept of "rebalancing" PN, so the plasma AA profile more closely resembles that of healthy infants. Our group's previous physiological studies found that this can partly be achieved using arginine supplementation. Increasing arginine content decreases the content of other AA, simultaneously reducing the mean plasma level of EAA and bringing plasma arginine closer to the reference range. Interestingly, a similar rebalancing effect was seen in the article by Poindexter et al. (126). Increasing the proportion of glutamine in the PN solution appeared to reduce plasma leucine, although the difference compared with the control was not significant. Rebalancing is the next step in a pathway necessary to design the next generation of neonatal AA formulations, specifically designed for early postnatal adaptation. Correcting the AA imbalance seems feasible, but further work is required to determine the optimum intakes before designing an RCT that tests for clinical benefit. The systematic review methodology offers a practical way to calculate these intakes and optimise the neonatal plasma AA profile.

It could be argued that until a consensus is reached on the correct reference plasma AA profile for a PN-dependent VPN, there is no reason to alter the formulation as this is a lengthy and costly process. Defining a reference plasma AA profile should be a major focus of nutrition research, as AA toxicity and deficiency both have the potential to completely undermine the current high-protein strategies aiming to accelerate early growth. In the case of leucine,

toxicity could have harmful implications on immune function and development. Future studies using AA solutions with a lower leucine content of 8-9g/100g AA are required. However, it seems pragmatic to develop a formulation that can achieve a plasma AA profile matching the profile of a healthy breastfed preterm infant for the interim period.

3.5.3. Analysis of bias factors

It was important to consider whether individual study design aspects could affect plasma leucine measurement. The first factor was the overall study design, and the articles were analysed in two groups, RCT versus non-RCT design. The three non-RCT studies are indicated using an asterisk (*) in Table 3.8. Statistical tests indicated that the mean difference was not significant, meaning the overall study design did not impact the plasma leucine level.

Another potential bias factor was the analytical method used for plasma AA analysis. Using Figure 3.4, the data points analysed by IEC tended to have a lower plasma leucine level compared with the six points analysed by HPLC, which cluster towards the higher plasma levels. However, a degree of variability was expected as literature findings suggested spectroscopy methods can vary in accuracy depending on the calibration of the instrument and reference method, resulting in slightly different measurements of plasma AA values (239,240) before you consider the differences between studies. Consequently, when designing a protocol for a PN formulation study, the methodology used for AA analysis should be selected carefully. The analytical method used may affect measurements, requiring adjustment to threshold values.

3.5.4. Quality appraisal

The quality assessment tool (QAT) highlighted substantial variation in the data collection and reporting between studies in this area, making data compilation and comparison more challenging. Other tools were considered for quality assessment, notably the Critical Appraisals Skills Programme (CASP) checklist for an RCT (241). However, 9 of the 12 articles in the systematic review were RCTs, and three had other study designs, including a non-randomised control trial and a crossover study. Consequently, the 12 papers could not be assessed using existing tools because these are usually tailored to a specific study design. A custom designed QAT enabled the assessment of the articles using the same set of questions. The QAT designed

for this review (attached in Appendix 4) appraises the study design, results and relevance of the paper in a similar manner to the CASP criteria. This QAT could guide protocol design for future neonatal PN studies.

The QAT gave evidence of some critical areas of weakness; less than half of the included articles had a clearly stated hypothesis, and a third reported using a power calculation to determine the appropriate sample size. Only half of the articles reported the route of PN administration and the duration of study PN. Furthermore, five papers failed to describe the method of AA analysis in sufficient detail, including information on the techniques, instruments or equipment used. These are crucial details that ensure accurate interpretation of findings, especially in the fields of neonatology and nutrition. The absence of essential information regarding the methods used reduces the reproducibility of the studies. Consequently, these findings should guide other researchers in this field to ensure better reporting in future studies.

The GRADE framework was used to rate the body of evidence presented by the systematic review at the outcome level. The risk of bias was serious, meaning the GRADE assessment was downgraded. Although 9 of the included studies were RCTs, 5 did not clearly explain the sequence generation and randomisation process in sufficient detail. Furthermore, allocation concealment and blinding were unclear in many studies.

Publication bias was considered moderate as several studies did not include a statement on funding or conflict of interest. Also, two studies were funded by the pharmaceutical companies whose parenteral AA solution was being tested, increasing the risk of bias. Another source of publication bias may be studies that found null results that went unpublished and were not captured by the search process. Whilst unresolvable and challenging to quantify, unpublished studies should be recognised as a possible contribution to publication bias.

The studies included had a variety of primary outcomes resulting in considerable inconsistency across the 12 studies. There was also a high level of heterogenicity from the different interventions and populations for each individual study. The mean gestational age for each study ranged from 25.7 to 31 weeks.

Similarly, the dataset demonstrates a low level of indirectness because whilst the articles included in the review studied the target population and reported the outcome of interest, the primary outcomes of many of the included studies were not in line with the primary outcome of the systematic review. This is most likely because few neonatal PN studies report plasma AA data as the primary outcome. Many studies included in this review focus on postnatal growth or the timing and advancement of AA in neonates, reporting plasma AA profiles as a secondary outcome analysis. Finally, the data also had high imprecision when assessing outcomes because of the wide range of reference values for plasma leucine concentration.

On the other hand, finding a dose-concentration relationship (Figure 3.3) uprated the certainty of the evidence. The ability to produce scatterplots establishing a linear relationship between leucine intake and plasma levels increased confidence in the body of evidence presented.

Due to crucial weaknesses in the GRADE domains discussed above, the certainty of evidence presented by this review would be categorised as low. Consequently, confidence in the effect estimate is limited, and the actual effect size may differ substantially from the estimation of optimal parenteral leucine intake presented by this review. The evidence is not robust enough to result in policy changes or changes to clinical practice. There is a requirement for further studies to increase the value of the evidence found.

3.5.5. Strengths and limitations

The main strength of this review was the ability to indirectly measure the relationship between leucine intake and plasma leucine level by calculating the absolute intake (total AA intake x percentage leucine content of AA solution). Consequently, estimating the threshold leucine intake required to achieve the desired plasma leucine level is possible. Premakumar et al. have already investigated the relationship between arginine intake and plasma arginine levels. This demonstrates that the approach works for excess AA provision in the case of leucine but also AA deficiency (215). Once a consensus is reached on the reference range for individual AA, this strategy could be replicated for the remaining AA, providing a practical way to rebalance PN formulation and optimise the neonatal AA profile using existing AA profile data. However, it is

unknown whether normalising the plasma AA will result in substantial clinical benefits, including optimised autophagy activation by reduced leucine intake.

This review considered 112 µmol/L as a desirable plasma leucine level based on the mean of a wide range of levels reported from various neonatal populations in published studies (Table 3.7). However, it is unknown whether this is the most appropriate target concentration. It is also important to remember that reference plasma leucine level should be reported as a range. Consequently, using only the mean value will exclude the many infants below the mean who are within the limits of the normal range, a limitation of this approach. However, 112 µmol/L was used to demonstrate how the regression equations could be used, rather than to make solid clinical recommendations. Furthermore, 112 µmol/L is close to the mean level of term breastfed infants (111.3 ± 27.3 µmol/L) (226), a substantially different population to VPN in terms of nutrition source and metabolic maturity. In reality, the target leucine level should perhaps have been closer to that of a preterm, human-milk-fed infant (87 ± 14 µmol/L) (228). These assumptions can only be tested and understood through further research. Consensus is required on the optimal plasma leucine before AA solutions could be reformulated. Nevertheless, the equations provided by this review allow those designing an AA formulation to modify their choice of target plasma leucine, an advantage of this approach.

A significant limitation was the small number of studies eligible for inclusion. Consequently, the relationship between leucine intake and plasma leucine level described by this review was based on an estimate from only 12 articles, some with sample sizes as small as 15 VPN. The low number of eligible studies likely resulted from a lack of plasma AA monitoring in clinical practice. Unfortunately, plasma AA profiles are not included in many audit cycles or quality improvement projects (QIP), which could provide the large volumes of clinical data needed to drive the improvement of commercial PN solutions.

The routine measurement of the plasma AA profile is not currently recommended in PN-dependent VPN by international nutrition guidelines (113). Therefore, the only source of plasma AA data is from research studies, and even then, a surprising number of large RCTs do not include plasma AA levels as an outcome. Recent studies investigating the plasma AA profile have tended to focus on the first 72 hours of life (63,122) rather than the second or third week

when VPN remain PN-dependent. Most PN research has focused less on leucine than other AAs, such as glutamine, arginine, tyrosine and threonine. One recommendation by this review would be to increase awareness of the importance of plasma AA monitoring and reporting in clinical practice. Whilst plasma AA monitoring does not provide individualised nutritional safety data for a VPN due to the slow laboratory turnaround time, it allows monitoring AA profiles in different neonatal cohorts. Whether this approach is feasible or even justifiable in secondary neonatal centres is unknown. However, it is worth considering until a more practical option becomes available.

A second drawback is that the review included neonates with gestational ages ranging from 23 weeks to 33 weeks. Including a small number of neonates greater than 32 weeks' gestation weakened the review further as not all the infants studied were very preterm. Furthermore, the metabolic and GI maturity of an extremely preterm neonate born at 23 weeks' gestation differs significantly from a 32-week neonate (242). Infants at either end of this range are not comparable, potentially resulting in higher leucine levels in the comparatively more mature VPN. However, a wide range of gestational ages was accepted for this review to increase the number of articles and VPN eligible for inclusion. In addition, many neonatal studies use 32 weeks as the upper limit of their inclusion criteria, meaning it was a pragmatic choice.

As discussed in Chapter 1, plasma AA levels are not accepted as an exceptionally reliable measure of AA status. This was a limitation of the systematic review design, and arguably single AA isotope studies would have been a better, albeit less practical, parameter for measuring individual plasma AA values (207). On the other hand, one benefit of a plasma AA profile over an isotope study is that they simultaneously assess all AA. They are also more accessible in a clinical setting. However, there is an obvious need for new technologies capable of rapidly measuring plasma AA concentrations in clinical practice. Real-time monitoring of a patient's nutritional status is essential when AA doses are increased. Infants may have rapidly changing AA requirements depending on gestational age, postnatal age, and illness severity (167). Nevertheless, the linear relationships shown by the dose-concentration graphs (Figure 3.3) suggest that plasma profiles may still be a clinically useful marker of adequate AA intake.

Finally, given the time-constraints of the MPhil project, it was not possible to complete a meta-regression. A meta-regression would describe how plasma leucine level is predicted according to other characteristics of the studies that may influence effect size, such as gestational age and the AA intake at the time of sampling. Additionally, larger studies such as the article by Poindexter et al. (n = 141) would have more influence on the relationship than smaller studies including Thornton and Griffin (n = 15). Completing a meta-regression is one way by which this work could be extended, and because meta-analyses are placed at the top of the hierarchy of evidence, it would present the best evidence for the relationship between leucine intake and plasma leucine level. In order to account for variability in gestational age, the gestations reported as median (IQR) need to be converted into mean and SEM to enable meta-regression (243).

3.6. Conclusion

The systematic review presents two critical findings. Firstly, it provides evidence of positive linear relationships between parenteral leucine intake (absolute intake and percentage content) and plasma leucine level. Overall, absolute leucine intake is a better predictor of plasma leucine level. However, both measures should be considered when designing a PN regimen. Secondly, this review has provided equations that can predict the plasma leucine level based on absolute leucine intake (mg/kg/d) or the proportion leucine content of a PN solution (%). These equations could be rearranged to predict the threshold leucine intake required for a predetermined plasma leucine level.

3.7. Implications for further research

Despite the limited number of eligible articles, this chapter reports important findings regarding parenteral leucine supply, which could be applied to reformulate PN solutions. Our methodology offers a systematic approach to rebalancing PN formulations using the existing evidence base. However, it seems reasonable to ensure a consensus on the reference ranges is reached first. Once the optimum content for each individual AA is determined, there is a need for a well-designed series of studies that rebalance the AA composition of PN solutions and test for any resulting clinical benefit. The next chapter of this thesis investigates this concept using a pilot transcriptomic analysis.

3.8. Additional work

An abstract for this systematic review of the literature was submitted to the Royal College of Paediatrics and Child Health (RCPCH) Conference 2022. It was successfully accepted for presentation as an e-poster. The abstract and corresponding poster can be seen in Appendix 6 and 7.

An additional abstract was submitted to the European Academy of Paediatric Societies (EAPS) Conference 2022 and was successfully accepted as an oral presentation. The abstract submitted is attached in Appendix 8.

Chapter 4: A Pilot Transcriptomic Analysis Exploring Changes in Autophagy-Related Gene Expression During Early Postnatal Life.

4.1. Introduction

Very preterm neonates (VPN) are PN-dependent for a bridging period whilst enteral feeding is established. For infants <29 weeks' gestation, the median length of PN dependence (>75% of all nutrition) is 15.6 days (22,23). PN dependency coincides with a crucial postnatal immune development period as VPN adapts from the sterile, protective uterine environment to survive in the colonised external world. Neonates, especially those born preterm, are among the highest-risk patient groups for mortality and morbidity secondary to infection (244).

The impact of different PN regimens on the risk of sepsis has been described in critically ill adults (143), as well as preterm (146) and term (145) infants. The latter, the PEPaNIC study, identified AA as the parenteral macronutrient most associated with poorer outcomes. The study authors speculated that the underlying mechanism might be improved autophagy activation following delayed PN. Given the proposed link between excess EAA provision, impaired autophagy through mTOR, and a deficient innate immune response, further and more detailed investigation is required to clarify the physiologic relationship between PN and early postnatal immune adaptation.

There are many groups which are studying multiple aspects of nutrition, however, there remains inadequate understanding of the biochemical changes and metabolic needs after birth in VPN. Transcriptomics, coupled with a bioinformatic interrogation of the resultant data, provides a sensitive platform through which to develop an understanding of the postnatal changes underpinning the early development in these infants, highlighting critical processes in postnatal adaption.

This thesis chapter examines existing transcriptomic data from the PAINT study to conduct a preliminary exploration of whether gene expression changes in mTOR-related genes between postnatal day 3 (D3) and day 10 (D10). The chapter also aims to evaluate the feasibility of transcriptomic analysis and multi-omics studies as an alternative methodology that can be

used in the future to understand the role of individual parenteral AA in immune system development.

4.2. A brief overview of the developing immune system

The developing immune system is a formidably complex topic. Fetal development begins at embryogenesis, with the haematopoietic system being amongst the first to emerge (245). However, like many other organ systems, the immune system is not fully functional at birth, placing neonates at an increased risk of infection compared to adults. Growing evidence suggests that the neonatal immune system is distinct and more dynamic than previously appreciated, not merely immature (246). Immune ontogeny during the early postnatal period is highly important given that severe infections cause significant neonatal mortality globally, accounting for 36% of neonatal deaths (247). Early postnatal immune adaptation is crucial for initial survival and shapes the immune response throughout life.

The increased susceptibility of preterm infants to infection is multi-factorial. Firstly, prenatal immune responses are biased against the production of Th1 cell proinflammatory cytokines, an effect which persists into the neonatal period. Increased inflammatory cytokines such as IFN-γ can induce apoptosis in fetal cells, increasing the risk of spontaneous abortion and preterm birth (248). Ex-utero, the continued suppression of inflammatory cytokines is suspected to be a protective adaption to the potentially overwhelming exposure to large amounts of new antigens in early life (249). Nevertheless, the price of ensuring intrauterine survival is the skewing of the neonatal immune response towards suppression.

During the period of greatest immune immaturity, neonates benefit from the placental transfer of maternal antibodies to the fetus, providing interim protection against vaccine-preventable infections during the first months of life (248). Transplacental transport of IgG occurs via a neonatal Fc receptor (FcRn). VPN receive substantially less maternal IgG, given that antibody transfer predominantly occurs during the third trimester (250), partially explaining the increased susceptibility to infections in the preterm population.

In addition, IgA antibodies, cytokines, complement proteins and commensal bacteria are transferred via colostrum and breast milk (37,251). These bioactive factors assemble the infant microbiota, the communities of symbiotic and commensal microbes colonising the host. Arguably, the relative immaturity of the neonatal immune system enables the acceptance of the microbiota without being overwhelmed by simultaneous exposure to many inflammatory stimuli. Microbiota development is an essential postnatal adaptation as it has a fundamental role in gut epithelial health, barrier function and the induction and calibration of the immune system (252). Thus, human breast milk provides a vertically transmitted, milk-oriented microbiota that aids immune development (253). VPN are often fed parenterally during the early postnatal immune adaptation period, so they do not receive this additional passive protection. They also risk microbial deprivation, a risk factor for various inflammatory and autoimmune diseases in later life (254).

A significant contributing factor to neonatal infection risk is the constant stream of medical interventions used extensively in neonatal units. IV lines, endotracheal tubes (ETT) and nasogastric (NG) tubes are routinely inserted, breaching the protective epithelial and mucosal barriers. Mucosal surfaces are a frequent entry point for pathogens meaning the development of mucosal immunity is a primary mechanism for infection prevention (255).

VPN are subject to unique immunological challenges. Protection against invading pathogens is achieved by the coordinated and interweaving reactions of the innate and adaptive arms of the immune response. Deficiencies of both innate and adaptive immunity partly explain the impaired neonatal immune defence, and the more premature the neonate, the more severe and prolonged the immunodeficiency (256). The impact of prematurity on the two responses is discussed in short below.

4.2.1. The Innate response

The non-specific, innate response is responsible for the primary immune defence mechanisms via the action of complement and phagocytosis-mediated killing of pathogens. Innate immunity involves inbuilt defence mechanisms, which do not require prior antigen exposure. Nevertheless, the innate immune system has reported significant quantitative and functional deficiencies. The general characteristics of neonatal innate immune cells are compared with their adult counterparts in Table 4.1.

Cell Type	Characteristics of neonatal cell type relative to adult cells	References
Neutrophils	- Smaller storage pool - Initial reduction in phagocytic ability - Reduced recruitment to sites of infection - Delayed NET response	(257,258)
Monocytes	- Higher levels in neonates than adults - Diminished capacity for antigen presentation - Lower cytokine production in response to stimuli - Reduced recruitment to sites of infection	(258,259)
Macrophages	- Low number of alveolar macrophages at birth - Reduced antigen processing and presentation - Delayed recruitment of monocytes and neutrophils to infection site	(260)
Natural Killer (NK) Cells	- Impaired ability to adhere to target cells - Reduced cytotoxic capacity	(261)
Dendritic Cells (DC)	- Reduced capacity to stimulate other immune cells - Reduced ability to produce IFN-α/β, important immune regulators	(262)

Table 4.1: Deficiencies of neonatal innate immune cells. Adapted from Korir et al. (263)

Likewise, the neonatal complement system is also underdeveloped. Complement proteins cannot be transferred across the placenta from mother to fetus. Therefore, levels of neonatal complement proteins are only 10-80% of those found in adults (264). Reduced complement levels are associated with impaired chemotaxis and deficient opsonisation.

4.2.2. The adaptive response

In contrast, the adaptive arm of the immune response is designed to eliminate specific pathogens and develop protective immunological memory. Both lymphocyte types can develop into memory cells that respond quickly when exposed to the same pathogen. However, the neonatal adaptive immune system is "antigen inexperienced", leaving infants highly susceptible to infection. Immune memory develops throughout life, and deficient levels of memory cells are detected in preterm neonates (265). Furthermore, deficiencies in the innate immune system can lead to a diminished adaptive immune response (263).

As discussed above, the neonatal immune response is characterised by minimal Th1 cell activity but an excess of anti-inflammatory Th2 function (266). The reduced neonatal capacity to produce an inflammatory Th1 response increases vulnerability to infections, and the bias toward Th2 cytokines cells confers a risk of developing atopic conditions in childhood (267). Neonates also demonstrate reduced numbers of Th17 cells, which are important in developing immunity to bacterial and fungal infections at mucosal surfaces (263).

Meanwhile, neonatal B cells are naïve, lacking antigen exposure with only a partially developed repertoire of surface Ig, part of the antigen receptor. Coupled with reduced B Cell receptor signalling, immature B lymphocyte function contributes to a significant deficiency in neonatal antibody production (245,263). In particular, the neonatal antibody response to polysaccharide antigens is weak, exemplified by the high infant mortality rate associated with infections caused by encapsulated bacteria (Streptococcus pneumoniae, Haemophilus influenzae and Neisseria meningitidis) (268).

To summarise, neonatal immune function is dampened and suffers from inexperience because of limited pathogen exposure in-utero, impairing the ability to respond effectively to infections. In the face of a deficient and rapidly maturing adaptive immune system, the comparatively more developed innate response is critical for the survival of neonates (263). The postnatal adaption period is a "window of opportunity", which, if utilised effectively, could induce a more potent immune response in vulnerable infants (269).

4.3. Autophagy, mTOR and the innate immune response

The neonatal subgroup analysis of the PEPaNIC study revealed that late initiation of PN in a group of critically ill, term neonates reduced the risk of a PICU-acquired infection compared with early initiation of PN (145). These findings sparked renewed concerns about early AA provision to very preterm infants, the population that forms the bulk of paediatric patients receiving PN. Determining the underlying mechanism was beyond the scope of the study, but impaired autophagy following the early initiation of PN seems a plausible explanation.

Autophagy is a mechanism of lysosomal-mediated protein degradation crucial in maintaining cellular homeostasis. In response to starvation, autophagy is accelerated to produce AA for gluconeogenesis (152). Given that autophagy is activated by fasting, including periods of anorexia induced by critical illness, it follows that delayed or insufficient neonatal PN would activate autophagy. Autophagy deficiencies have been documented in humans with a prolonged critical illness (161) and parenterally fed rabbits (162), the severity of the deficiency correlated with the amount of AA infused.

Over the last few years, autophagy activity has been reported in several classes of immune cells, such as macrophages, DCs and lymphocytes (270). Specific roles for autophagy have been in the immune system, ranging from pathogen recognition (271), modulation of the inflammatory response (272), antigen presentation (273) and the regulation of T cell activation (270,274), all fundamental elements of the innate immune response.

Autophagy is negatively regulated by the nutrient-sensing mTOR kinase, a master cellular growth and metabolism regulator (275). mTOR complex 1 (mTORC1) is associated with the regulation of protein synthesis and is activated in two ways: firstly, by insulin or growth factor signalling and secondly, by AA. The pathway through which insulin activates mTOR is well described and is outlined in Figure 4.1. The ultimate phosphorylation of ribosomal protein S6 stimulates translation and protein synthesis (165). The mechanism by which AA stimulate protein synthesis through mTOR is less well understood, but it is widely accepted that the pathway is independent of the insulin signalling cascade (276,277).

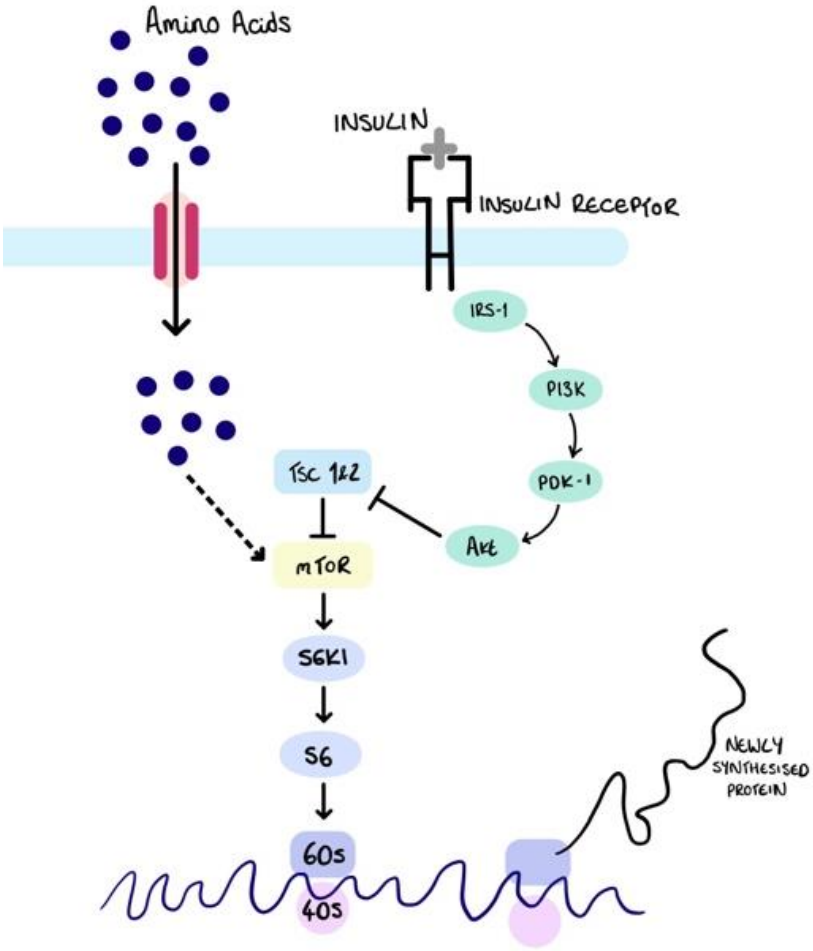


Figure 4.1: Summary of the mTOR signalling pathway adapted from Columbus et al. (165)

4.3.1. Immunonutrition

The potential to modulate immune system activity with specific nutrients is termed immunonutrition, and awareness of the importance of the concept is increasing. Previous studies have shown clinical benefits from optimising single immunonutrients, glutamine and arginine, both deficient in PN-dependent VPN. Enteral glutamine supplementation was reported to decrease the incidence of hospital-acquired sepsis in premature neonates (278) and pneumonia, bacteraemia, and severe sepsis in other critically ill patient groups (279,280). Evidence is also emerging that arginine metabolism is a crucial regulator of innate and adaptive immune responses (281).

On the other hand, leucine is a potent activator of the mTOR pathway (169) but is provided in excess by PN solutions (124). An argument can be made that if the immune response can be augmented by supplementing deficient AA, reducing the plasma levels of overprovided EAA (including leucine) may have an unmasked effect on innate immunity through improved autophagy activation. Many questions remain unanswered, and more evidence is required to support this physiological explanation for the PEPaNIC study findings. Nevertheless, the proven influence of autophagy on critical aspects of innate immunity makes it an attractive target for attempts at nutritional therapies which enhance neonatal immunity. An improved understanding of the immunological mechanisms at play during the neonatal period may help develop nutritional strategies to manipulate the immune response and prevent VPN from being so vulnerable to infection.

4.4. The PAINT Study

4.4.1. Background

The PAINT (Preterm Arginine INTake) study was the first in a series of physiological studies exploring the effects of increased parenteral arginine intake on biological pathways affecting immune function in VPN. The study was designed to investigate the effect of supplementary arginine using a multi-omics approach that included transcriptomics, metabolomics, plasma AA levels, and blood ammonia testing to assess a nutritional intervention in relation to the developmental changes occurring in VPN over the first 10 days of post-natal adaption.

Transcriptomics is the study of the transcriptome, the study of all the RNA transcripts, both coding and non-coding. This includes the transcription and expression levels, functions, locations, trafficking, and degradation (282). Microarray is a technique which permits the analysis of the expression of a large number of genes simultaneously. The measurements can then be compared between time points to identify changes in gene expression (283). Transcriptomics analysis could be used to understand the gene expression changes underpinning postnatal immune adaptation in VPN.

The primary objective of the PAINT study was to determine alterations in gene expression between D3 and D10 in infants receiving additional arginine supplementation (12% versus 15% arginine) compared with unsupplemented infants. Arginine depletion has been shown to affect T cell function, and immune signalling may also be affected by reduced NO synthesis in response to hypoarginemia (197,284,285). Initial transcriptomic analysis of the PAINT data confirmed that the early days of life are a critical period of immune system development in VPN. Three immune processes were significantly upregulated: neutrophil signalling and activation, phagocytosis by monocytes and macrophages and T cell activation.

In the context of the PEPaNIC study findings, it was appropriate to revisit the PAINT data to explore whether there was any evidence of gene expression changes specifically in mTOR-related genes between D3 and D10. This preliminary analysis tested the hypothesis that high plasma EAA levels, indicating potential EAA 'toxicity', result in changes to mTOR gene expression, which may impair autophagy and innate immunity in VPN.

We also aimed to determine the feasibility of further transcriptomic analysis as part of future physiological studies in PN-dependent VPN.

4.4.2. Methods

The study received all the necessary ethical and regulatory approvals. This was a single centre, unblinded study with non-random allocation of standard treatment or arginine supplementation (PAINT). The infants received the standard PN at LWH, which uses Vaminolact (Fresenius Kabi), an AA formulation known to provide excess EAA, including leucine (124).

Inclusion criteria:

Infants were born <29 weeks' gestation and/or <1200g. Infants were admitted to the NICU at LWH within 48 hours of birth in Autumn 2016.

Exclusion criteria:

a) Infants who are unlikely to survive the first week after birth
b) Infants with early onset infections (<72 hours).
c) Infants known (or suspected to have) a diagnosis of inborn error of metabolism or serious liver dysfunction.
d) Parents who are unable to give informed consent.

Eligibility and consent:

The parent(s)/guardian(s) of each potentially eligible patient was approached when the baby was stabilised on the neonatal unit. Consent was sought within 72 hours of birth. When clinical circumstances permitted, parents were approached antenatally. The investigator explained the study fully to the patient's parent(s)/guardian(s) using a patient information leaflet.

Blood sampling and processing:

The first study-related blood samples were collected on D3, with the final samples taken on D10. 200µl of whole blood was collected for RNA extraction and microarray analysis alongside blood for routine biochemistry and plasma AA levels. Immediately following sampling 600µl of Blood RNA Buffer (ZR Whole-blood RNA MiniPrep™) was added, as per manufacturer's instructions (Zymo Research, USA), and the sample was stored at -80°C. Samples were initially processed and stored at LWH prior to batch transport to the Institute in the Park, Alder Hey Children's Hospital for RNA extraction.

Following RNA extraction, microarray analysis was conducted by the Centre for Genomic Research at the University of Liverpool (UoL). Expression data were normalised, logged and analysed using Significance Analysis of Microarrays (SAM) within the statistical software R. Then, pathway analysis was conducted using Ingenuity Pathway Analysis (IPA) software (QIAGEN Inc.). The pathway analysis was used to identify gene expression changes related only to mTOR, focusing the huge volume of microarray data. The candidate, KD, did not contribute to the data collection for the first PAINT study. However, to gain an understanding of the sampling, storage and analysis techniques, KD contributed to the real-time data collection for the sister PAINT18 study.

4.4.3. Results

Only the eight unsupplemented (control) infants were included in this secondary analysis, to introduce the statistical and metabolic pathway analyses required to interpret large transcriptomic data sets but in small patient numbers. The control infants received standard PN only. Infants who received supplementary arginine infusions were excluded from this analysis. Infants had a mean (SD) gestational age at birth of 26+4 weeks' (± 1.8) and a mean birth weight of 871g (± 194). There were five male and three female infants in the group. All the mothers had received antenatal steroids.

Table 4.2 shows the total parenteral leucine intake (mg/kg) for each infant in the 48 hours preceding the day 10 sample. The initial PAINT data collection only captured the arginine intakes from each infant. However, using the SCPC document in Appendix 1, a conversion factor of 1.7 was calculated. The mean (SD) parental leucine intake was 374.0 (112.7) mg/kg/72h.

Infant	Parenteral Arginine Intake (mg/kg/72h)	Parenteral Leucine Intake (mg/kg/72h)
1	253.0	430.2
2	265.7	451.7
3	156.6	266.2
4	258.3	439.1
5	80.8	137.4
6	246.4	418.9
7	238.4	405.3
6	260.9	443.6

Table 4.2: Parenteral leucine intakes for the eight control PAINT infants

The PAINT study analysed 34,758 genes. The pathway analysis identified five genes that directly interacted with the mTOR pathway and were significantly upregulated on D10 with respect to D3 (Figure 4.2). The primary function of each gene was investigated using GeneCards, a searchable, integrative human gene database (286) alongside any available literature.

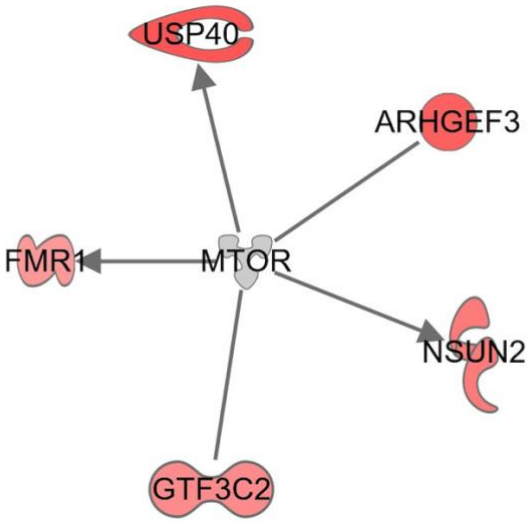


Figure 4.2: Representation of the direct interactions of mTOR with the transcriptomic data from the PAINT study. Red indicates the gene was upregulated on D10 with respect to D3.

Table 4.3 summarises the function of each gene, alongside the fold change, indicating the ratio of change between D3 and D10 samples and the q-statistic. Q-values are a widely used statistical method for estimating false discovery rate (FDR), a conventional significance measure in the analysis of genome-wide expression data (287).

Gene	Function	Fold change	Q value
USP40	Ubiquitin Specific Peptidase 40 Modifying cellular proteins by ubiquitin is an essential regulatory mechanism controlled by ubiquitin-conjugating and deconjugating enzymes.	2.10	4.90
ARHGEF3	Rho Guanine Nucleotide Exchange Factor 3 Acts as guanine nucleotide exchange factor (GEF) for RHOA and RHOB GTPases, both of which have a role in bone cell biology.	1.97	4.16
NSUN2	Encodes a methyltransferase that catalyses the methylation of cytosine to 5-methylcytosine (m5C) in the tRNA(Leu)(CAA) precursors. This modification stabilises the anticodon-codon pairing to translate the mRNA correctly.	1.63	4.90
GTF3C2	Required for RNA polymerase mediated transcription.	1.43	4.90
FMR1	Fragile X Messenger Ribonucleoprotein 1 Codes for the FMRP gene, which is essential for cognitive development and synaptic protein synthesis. Suggested role in the nucleocytoplasmic shuttling of mRNA. Mutations of this gene can lead to fragile X syndrome	1.26	4.90

Table 4.3: Summary of the functions of the five mTOR genes that were upregulated between D3 and D10 in the Summaries adapted from genecards.org (286). PAINT study. No genes were downregulated meaning the expression of an estimated 1160 mTOR genes was unchanged.

4.4.4. Discussion

In the present study, the pathway analysis identified five isolated mTOR-related transcripts upregulated between D3 and D10 of life. We did not identify any transcripts which were significantly downregulated. As part of a different study, Yates et al. compiled a list of 1165 mTOR-related genes (288), meaning an estimated 1160 mTOR genes demonstrated no change. This indicates that our analysis shows a minimal change in total mTOR-related gene expression, with many transcripts unchanged. In contrast, the currently unpublished pathway analysis of the initial, broader analysis of the PAINT transcriptomics showed that the affected genes are meaningfully clustered in key (adaptive) immune pathways. A rapid change in the adaptive response is expected given the colossal increase in antigen exposure and adaptive immune cell function gain that occurs soon after birth. Early postnatal innate immune system maturation is less dramatic, given that the mechanisms are developed antenatally and are functional by birth (141,245).

The lack of significant change in mTOR, even when leucine is overprovided by Vaminolact, makes a leucine-based immunonutrition intervention in the early postnatal adaptation period less plausible. Finally, this analysis implies that the pathophysiological explanation for superior clinical outcomes following delayed PN (PEPaNIC) is unlikely to be improved autophagy activation by delayed AA initiation. However, this is a preliminary finding which requires further investigation.

When considering immunonutrition, it is also important to consider the potential adverse effects of excessive innate immune system activation in VPN through immunonutrition-based intervention. In addition to a protective role against pathogenic organisms, the immune system also ensures tolerance to the microbiome and commensal bacteria. The neonatal period represents a critical window in which the immune system must balance efficient pathogen defence without being overwhelmed by exposure to a flood of commensal microbes and environmental antigens. Dampening the immune response ensures microbiota establishment occurs without overt inflammation and collateral damage to the body in response to benign, non-infectious antigens. Dysregulated innate immune responses contribute to the aetiology of multiple neonatal complications, such as bronchopulmonary dysplasia (BPD) and NEC

(289,290). Clearly, immunonutrition should be thoroughly researched using a range of methodologies (analysis of existing data and further multi-omics work) and approached cautiously in critically ill patients, particularly VPN, who may respond differently than older children and adults.

Ultimately, the PEPaNIC study findings mandated better understanding of the impact of PN on the postnatal development of the immune system in VPN. This pilot PAINT study analysis has partly explored this. Further transcriptomic work would strengthen conclusions about using AA interventions as a form of immunonutrition. Once we understand the broader mechanisms better, PN formulations can be safely manipulated and tested to investigate the potential therapeutic benefits of AA as immunonutrients, hopefully reducing VPN vulnerability to infection.

4.4.5. Strengths and limitations

PAINT was a small exploratory physiological study with limitations, particularly the small sample size. Arguably, changes in gene expression from 8 relatively heterogenous infants with a similar patient phenotype and clinical course are insufficient to draw firm conclusions regarding the impact of PN on mTOR and autophagy. The changes in mTOR gene expression may have been part of a normal physiological adaptation process to extrauterine life, unrelated to PN. Additionally, because leucine was similarly provided to all infants, the analysis could not assess the extent to which different leucine levels modify transcripts. A potential avenue for further research would be detailed bioinformatics and systems biology work in a larger cohort of infants receiving different leucine intakes, enabling investigation of whether variable leucine intakes can affect transcripts directly or downstream.

A second limitation was that all the participants' mothers had received a course of antenatal steroids. Antenatal steroid therapy is thought to have a short-term influence on the expression of a limited number of genes and gene pathways in preterm infants (291). However, administering maternal corticosteroids is standard care when preterm birth is anticipated. Therefore, the gene expression changes reported likely represents neonatal populations in other populations with developed healthcare systems.

Furthermore, by comparing D3 and D10, the transcriptomic analysis could only offer a snapshot, unable to capture all of the likely postnatal transformations occurring in VPN. Due to the complexity, cost and limitations in the volume of blood that can be safely collected from VPN, repeat transcriptomic analysis between other time points was not possible in this small study. Nevertheless, we still have much to learn about how PN affects VPN development. Further research is needed to extend our understanding of the relationship between nutrition, metabolic adaptation and the maturing immune system.

On the other hand, a strength of this study is that through the pathway analysis, we could focus on a small subset of autophagy-related target genes. The analysis provided a deeper insight into the impact of AA on early postnatal immune adaptation compared with the simple infection incidence comparison made in the SCAMP data analysis (Chapter 2).

Moreover, alongside the transcriptomics, the PAINT study conducted a metabolomic analysis of plasma, urine and faeces samples collected from the recruited infants. Metabolomics is the study of metabolites, the small molecule substrates, intermediates and products of cell metabolism. Metabolomic analyses enable the investigation of a wide range of metabolic pathways (for example, intermediates in the urea cycle in the context of arginine), providing data not available from routine biochemical analysis (292). Previously, research aiming to advance understanding of these fundamental metabolic processes has been highly problematic due to VPN's small size and fragility. However, transcriptomics and metabolomics analyses are now possible on micro-samples safely obtainable from VPN. Preliminary analysis of the metabolomic results suggest that infants who respond to arginine supplementation exhibit different metabolomic profiles than unsupplemented infants.

These results highlight the feasibility and importance of using a multi-omics approach alongside clinical and biochemical outcomes. A multi-omics analysis offers an integrated and comprehensive insight into the broader effects of different AA interventions on biological pathways (metabolic, inflammatory and immune-related), which require investigation when modifying PN. These studies generate a plethora of data, which, when carefully integrated, can elucidate biological processes, molecular functions and interactions. The initial PAINT study directed us towards important immune pathways likely to change, enabling focus on these

pathways in future studies. Hence, the next PAINT study (PAINT18) was designed to investigate the hypothesis that arginine supplementation will result in changes in gene expression consistent with changes in T cell function and associated inflammatory pathways. This demonstrates how multi-omics analysis in VPN enables novel insight into critical processes in postnatal development and provides direction for future research.

Further physiological studies in PN-dependent infants are required to establish the clinical and biochemical importance of optimising single AA immunonutrients to impaired immune function and suboptimal postnatal immune adaptation. However, only robustly designed studies can combine single-omics datasets in a meaningful manner and generate convincing conclusions. The challenges of multi-omics research are abounding and are discussed elsewhere (293,294). Nevertheless, multi-omics remains an important tool that should be applied to many areas of neonatal research, including understanding postnatal adaptation concerning nutrition.

4.5. Conclusion

Our pilot exploratory analysis showed minimal and isolated changes in five mTOR-related gene expression between D3 and D10 of life. Investigating PN with less leucine or other EAA is unlikely to affect mTOR gene expression, autophagy, and subsequently the innate immune system substantially, an important preliminary negative finding in the VPN population considering the PEPaNIC proposals. The evidence to support delaying PN in preterm neonates remains weak. For the field of neonatology, further investigation is required to improve understanding surrounding how inadequate nutrition affects susceptibility to infection. This could include a well-implemented multi-omics approach that fully explores the role of AA, such as leucine and arginine, in postnatal immune system development.

4.6. PAINT18

PAINT18 is the latest in the series of physiological studies continuing to explore the effect of increased parenteral arginine intake on immune function in very preterm infants. The study is part of an effort to rebalance neonatal PN, correcting CEAA deficiency and simultaneously addressing the excess provision of EAA. The previous studies (PAINT and PAINT-NH4) found that even with a 15% arginine supplement, VPN do not achieve adequate plasma arginine levels, meriting further investigation. The primary objective of PAINT18 is to examine changes in gene expression in infants receiving 18% arginine supplementation between day 3 and day 30 of life. The protocol for PAINT18 can be seen in appendix 9.

The time constraints of an MPhil prevent a student from undertaking their own large clinical study. Hence, this thesis explored the effect of individual AA intakes on clinical infection risk and early postnatal metabolic adaptation in VPN using data from previous local neonatal PN studies. However, PAINT18 is currently recruiting at LWH. As part of this project, KD has observed the recruitment process, appreciating the unique challenges of obtaining informed antenatal or postpartum consent. KD witnessed the collection of blood samples for plasma AA analysis, noting the small volumes available alongside routine clinical blood samples and the methods used to extract and store RNA for analysis.

Furthermore, KD contributed to real-time data collection for PAINT18, providing a practical understanding of the methodology used in the SCAMP study (the subject of Chapter 2), which used the same principles to calculate nutritional intakes from multiple sources. Obtaining accurate nutritional data requires detailed fluid data collection. Whilst parenteral AA and lipids only have one source, glucose has multiple fluid sources as most drugs are delivered in glucose. Finally, participation in PAINT18 enhanced KD's understanding of biochemical and infection monitoring limitations during PN administration to VPN.

Chapter 5: General discussion

5.1. Summary of findings

As described in Chapter 1, PN is part of the standard care for a VPN. Despite extensive use, the optimal AA formulation for PN solutions has not yet been determined, a factor likely to contribute toward postnatal nutritional deficits and subsequent long-term growth and neurodevelopmental failure. A longstanding body of plasma AA evidence suggests an imbalance between EAA and CEAA provision by neonatal PN formulations. However, the commercial solutions used today have not been changed for more than 30 years to address this issue. The recent evidence from the PEPaNIC study associating parenteral AA with an increased risk of infection in term neonates creates further anxiety about early, high dose AA provision to VPN. The chapters of this thesis use different methodologies to explore these concerns, with a particular focus on leucine, an EAA known to inhibit autophagy through the mTOR pathway. Autophagy has an emerging role in the innate immune response.

The SCAMP data analysis in Chapter 2 demonstrated no significant difference in total parenteral AA intake between infants who developed LOS and those who did not. Comparison of the plasma EAA levels between groups showed no evidence of greater leucine overprovision in infants who developed LOS or NEC. However, infection and NEC appear to be associated with aggravation of CEAA deficiency, including arginine and glutamine. Correcting the deficiency of these particular CEAA appears a bigger priority and would have the additional benefit of proportionally reducing plasma EAA levels.

Thereafter, the systematic review in Chapter 3 collected existing plasma AA profile data to quantify the overprovision of leucine, providing dose-concentration gradients which could be used as a starting point to rebalance PN.

Finally, Chapter 4 explored the development of the neonatal innate immune system regarding PN, mTOR and autophagy. A pilot transcriptomic analysis showed minimal and isolated mTOR gene expression changes during the early postnatal adaption period. Investigating PN with

reduced leucine and other EAA appears unlikely to have substantial clinical effects on the innate immune system following changes in mTOR gene expression.

Overall, the preliminary evidence presented by this thesis does not support the hypothesis that EAA overprovision in PN-dependent VPN results in an increased risk of neonatal infection due to deficient autophagy. This is an important negative considering the anxiety generated by the PEPaNIC findings surrounding parenteral AA provision to VPN. The evidence to support delaying PN in preterm neonates remains weak.

5.2. Implications for clinical practice

One apparent recommendation for clinical practice would be to increase awareness of the importance of routine plasma AA monitoring and reporting, particularly in secondary centres. Establishing a larger dataset to assess average AA levels amongst the VPN population is especially important whilst a consensus is being reached on the appropriate reference ranges for designing an optimal neonatal PN solution. Hopefully, this thesis and later publications or presentations of our work will increase awareness of the issue.

5.3. Implications for research

This thesis has provided a good foundation in neonatal nutrition with multiple avenues for further study. Given that the composition of PN is modifiable, Chapter 2 indicates a need for future studies exploring the effectiveness of rebalancing deficient AA as a form of immunonutrition and defining the optimal composition. Regarding leucine, Chapter 3 suggests that the leucine content of neonatal PN should be reduced to 8-9g/100g AA to achieve reference plasma AA levels. Furthermore, the systematic review methodology offers a practical contribution toward rebalancing PN formulations using the existing evidence base, which could be replicated for the remaining AA. However, any formulation changes must account for solubility and stability issues that must be tackled early.

Chapter 4 highlighted the feasibility and importance of using a multi-omics approach to rebalance PN. Similar to the PAINT study, future neonatal PN studies should include metabolomics and transcriptomics, alongside clinical and biochemical outcome analysis, to

determine whether normalising the plasma AA will result in substantial benefits. The multi-omics analysis offers a sensitive platform for understanding the broader physiological changes (metabolic, inflammatory and immune) underpinning early postnatal adaptation.

The findings from smaller, physiological PN studies (similar to the PAINT studies) could stimulate large-scale clinical trials to license the next generation of AA formulations, specifically designed for optimal early postnatal metabolic adaptation in VPN. However, a consensus is required first on the most appropriate reference ranges to determine the ideal balance of EAA, NEAA, and CEAA for the preterm infant while considering functional AA requirements. Researchers might use the quality assessment tool created for the systematic review to guide protocol design for future neonatal PN studies, ensuring better quality and reporting of outcomes.

5.4. Limitations

Inevitably, each chapter had a number of limitations due to the niche nature of the research. In Chapter 2, multiple statistical tests were used on the SCAMP data, given that 20 individual AA were analysed. In addition, the infection group had a significantly lower birth weight than the no-infection group, a potential confounding variable. Future studies investigating plasma AA levels should be designed to investigate one individual AA as the primary outcome to minimise the impact of multiple testing.

Secondly, an important limitation of the systematic review (Chapter 3) was the small number of studies eligible for inclusion. Consequently, the review could not focus on a narrower and directly comparable range of gestational ages to accurately reflect the very preterm population. The data collected from the systematic review was also heterogeneous, preventing meta-analysis. As discussed above, increasing the awareness of the importance of plasma AA monitoring would expand the existing plasma profile dataset. Then, a meta-analysis could be conducted to strengthen the systematic review findings.

Chapters 1 and 2 also highlight the weaknesses of plasma AA profiles as a measure of AA status. Plasma levels represent a fine balance between AA intake, protein synthesis, degradation and excretion, and metabolic pathway activity for functional AA, including leucine. However, an AA profile remains the most clinically accessible measure of single AA deficiency or toxicity.

The transcriptomic analysis conducted in Chapter 4 was limited by the small sample size, which prevented any firm conclusions regarding the impact of PN on mTOR and autophagy. Further and more detailed bioinformatics assessment would enhance this work.

Finally, the overall project and participation in the PAINT18 study have highlighted the challenges and limitations of neonatal research. The complexity of designing an optimal neonatal parenteral AA formulation is also evident. However, this thesis and other ongoing work by our group provide a clear direction for research that will hopefully ensure VPN eventually receive an optimal AA formulation in terms of quantity and quality.

5.5. Concluding remarks

On the basis of this preliminary work, EAA overprovision does not confer an increased infection risk in VPN due to deficient autophagy, contradicting the suggestions made by the PEPaNIC study. However, the overall thesis has emphasised the potential clinical importance of designing a balanced parenteral AA solution, possibly using AA as immunonutrients to support postnatal immune development. We made important recommendations for future research, developing a neonatal parenteral AA formulation that maximises clinical benefits without causing metabolic or infection-related harm to VPN. Achieving this goal will require a sequence of systematic reviews followed by well-designed physiological studies that implement a multi-omics approach.

References

1. Gargasz A. Neonatal and pediatric parenteral nutrition. AACN Adv Crit Care [Internet]. 2012 Oct [cited 2022 Mar 8];23(4):451–64. Available from: https://pubmed.ncbi.nlm.nih.gov/23095971/

2. Mitton SG. Amino acids and lipid in total parenteral nutrition for the newborn. J Pediatr Gastroenterol Nutr [Internet]. 1994 [cited 2022 Mar 8];18(1):25–31. Available from: https://pubmed.ncbi.nlm.nih.gov/8126614/

3. Barnett MI, Pertkiewicz M, Cosslett AG, Mühlebach S. Basics in clinical nutrition: Parenteral nutrition admixtures, how to prepare parenteral nutrition (PN) admixtures. Eur e-Journal Clin Nutr Metab [Internet]. 2009 Jun 1 [cited 2022 Mar 16];4(3):e114–6. Available from: http://clinicalnutritionespen.com/article/S1751499109000031/fulltext

4. Helen Mactier (Consultant Neonatologist G by, Committee) BE, By O group members (self-nominated from B membership and approved, Committee): E, Dr Shri Babarao (Consultant Neonatologist W, Dr Jennifer Birch (Consultant Neonatologist L& D, et al. British Association of Perinatal Medicine The Provision of Parenteral Nutrition within Neonatal Services - A Framework for Practice April 2016. Br Assoc Perinat Med. 2016;(April):1–27.

5. Birth characteristics in England and Wales - Office for National Statistics [Internet]. [cited 2022 Mar 8]. Available from: https://www.ons.gov.uk/peoplepopulationandcommunity/birthsdeathsandmarriages/livebirths/bulletins/birthcharacteristicsinenglandandwales/2019

6. Prematurity statistics in the UK | Bliss [Internet]. [cited 2022 Mar 8]. Available from: https://www.bliss.org.uk/research-campaigns/neonatal-care-statistics/prematurity-statistics-in-the-uk

7. Holmgren PA, Högberg U. The very preterm infant - a population-based study. Acta Obstet Gynecol Scand. 2001 Jun;80(6):525–31.

8. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. Lancet [Internet]. 2008 Jan 5 [cited 2022 Mar 8];371(9606):75–84. Available from: http://www.thelancet.com/article/S0140673608600744/fulltext

9. Romero R, Espinoza J, Kusanovic JP, Gotsch F, Hassan S, Erez O, et al. The preterm parturition syndrome. BJOG [Internet]. 2006 Dec [cited 2022 Mar 8];113 Suppl 3(Suppl 3):17–42. Available from: https://pubmed.ncbi.nlm.nih.gov/17206962/

10. Dayal S, Hong PL. Premature Rupture Of Membranes. StatPearls [Internet]. 2021 Nov 2 [cited 2022 Jun 22]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK532888/

11. Helenius K, Sjörs G, Shah PS, Modi N, Reichman B, Morisaki N, et al. Survival in Very Preterm Infants: An International Comparison of 10 National Neonatal Networks. Pediatrics [Internet]. 2017 Dec 1 [cited 2021 Nov 16];140(6). Available from: https://pubmed.ncbi.nlm.nih.gov/29162660/

12. Costeloe KL, Hennessy EM, Haider S, Stacey F, Marlow N, Draper ES. Short term outcomes after extreme preterm birth in England: comparison of two birth cohorts in 1995 and 2006 (the EPICure studies). BMJ [Internet]. 2012 Dec 4 [cited 2022 Jun 26];345(7886). Available from: https://www.bmj.com/content/345/bmj.e7976

13. Liu L, Oza S, Hogan D, Perin J, Rudan I, Lawn JE, et al. Global, regional, and national causes of child mortality in 2000–13, with projections to inform post-2015 priorities: an updated systematic analysis. Lancet. 2015 Jan 31;385(9966):430–40.

14. Manuck TA, Rice MM, Bailit JL, Grobman WA, Reddy UM, Wapner RJ, et al. Preterm neonatal morbidity and mortality by gestational age: a contemporary cohort. Am J Obstet Gynecol [Internet]. 2016 Jul 1 [cited 2022 Mar 8];215(1):103.e1-103.e14. Available from: https://pubmed.ncbi.nlm.nih.gov/26772790/

15. Colvin M, McGuire W, Fowlie PW. Neurodevelopmental outcomes after preterm birth. BMJ [Internet]. 2004 Dec 9 [cited 2022 Mar 8];329(7479):1390–3. Available from: https://www.bmj.com/content/329/7479/1390

16. Mahoney AD, Jain L. Respiratory disorders in moderately preterm, late preterm, and early term infants. Clin Perinatol [Internet]. 2013 Dec [cited 2022 Mar 8];40(4):665–78. Available from: https://pubmed.ncbi.nlm.nih.gov/24182954/

17. Behrman RE, Butler AS. Preterm Birth: Causes, Consequences, and Prevention. Preterm Birth Causes, Consequences, Prev [Internet]. 2007 May 23 [cited 2022 Mar 8];1–772. Available from: https://pubmed.ncbi.nlm.nih.gov/20669423/

18. Mosca F, Giannì ML, Roggero P, Menis C, Morlacchi L, Liotto N, et al. Critical questions on nutrition of preterm infants. J Pediatr Neonatal Individ Med [Internet]. 2017 Jun 5 [cited 2022 Jul 7];6(2):e060203–e060203. Available from: https://jpnim.com/index.php/jpnim/article/view/060203

19. Neu J. Is it time to stop starving premature infants? J Perinatol 2009 296 [Internet]. 2009 May 27 [cited 2022 Apr 11];29(6):399–400. Available from: https://www.nature.com/articles/jp200946

20. Kashyap S, Schulze KF. Energy requirements and protein-energy metabolism and balance in preterm and term infants. Neonatal Nutr Metab Second Ed [Internet]. 2006 Jan 1 [cited 2022 Mar 9];134–46. Available from: https://www.cambridge.org/core/books/neonatal-nutrition-and-metabolism/energy-requirements-and-proteinenergy-metabolism-and-balance-in-preterm-and-term-infants/E5D98490B9F965EFA940D505844A2B08

21. American Academy of Pediatrics, Committee on Nutrition. Nutritional needs of low-birth-weight infants. Pediatrics. 1977 Oct;60(4):519–30.

22. Tan MJ, Cooke RW. Improving head growth in very preterm infants - A randomised controlled trial I: Neonatal outcomes. Arch Dis Child Fetal Neonatal Ed [Internet]. 2008 [cited 2021 Nov 30];93(5):337–41. Available from: http://fn.bmj.com/

23. Morgan C. Early amino acid administration in very preterm infants: Too little, too late or too much, too soon? Semin Fetal Neonatal Med [Internet]. 2013 Jun [cited 2022 Feb 28];18(3):160–5. Available from: https://pubmed.ncbi.nlm.nih.gov/23490859/

24. Van Den Akker CHP, Te Braake FWJ, Weisglas-Kuperus N, Van Goudoever JB. Observational outcome results following a randomized controlled trial of early amino acid administration in preterm infants. 2014 Dec 11 [cited 2021 Oct 12];59(6):714–9. Available from: https://pubmed.ncbi.nlm.nih.gov/25187104/

25. Zeitlin J, Ancel PY, Saurel-Cubizolles MJ, Papiernik E. The relationship between intrauterine growth restriction and preterm delivery: an empirical approach using data from a European case-control study. BJOG [Internet]. 2000 [cited 2022 Jun 30];107(6):750–8. Available from: https://pubmed.ncbi.nlm.nih.gov/10847231/

26. Carver TD, Quick AA, Teng CC, Pike AW, Fennessey P V., Hay WW. Leucine metabolism in chronically hypoglycemic hypoinsulinemic growth- restricted fetal sheep. Am J Physiol - Endocrinol Metab. 1997;272(1 35-1).

27. Hay WW, Lucas A, Heird WC, Ziegler E, Levin E, Grave GD, et al. Workshop Summary: Nutrition of the Extremely Low Birth Weight Infant. Pediatrics [Internet]. 1999 Dec 1 [cited 2022 Apr 18];104(6):1360–8. Available from: /pediatrics/article/104/6/1360/62721/Workshop-Summary-Nutrition-of-the-Extremely-Low

28. Abdel-Hady H, Nasef N, Shabaan AE, Nour I. Caffeine therapy in preterm infants. World J Clin Pediatr [Internet]. 2015 [cited 2022 Apr 20];4(4):81. Available from: /pmc/articles/PMC4637812/

29. Tijsseling D, Wolbeek M ter, Derks JB, De Vries WB, Heijnen CJ, Van Bel F, et al. Neonatal corticosteroid therapy affects growth patterns in early infancy. PLoS One [Internet]. 2018 Feb 1 [cited 2022 Apr 20];13(2). Available from: https://pubmed.ncbi.nlm.nih.gov/29432424/

30. Dinerstein A, Nieto RM, Solana CL, Perez GP, Otheguy LE, Larguia AM. Early and aggressive nutritional strategy (parenteral and enteral) decreases postnatal growth failure in very low birth weight infants. J Perinatol 2006 267 [Internet]. 2006 Jun 27 [cited 2022 Apr 11];26(7):436–42. Available from: https://www.nature.com/articles/7211539

31. Weinstein MR, Oh W. Oxygen consumption in infants with bronchopulmonarydysplasia. J Pediatr [Internet]. 1981 Dec 1 [cited 2022 Apr 18];99(6):958–61. Available from: http://www.jpeds.com/article/S0022347681800327/fulltext

32. Wilson D, McClure G. Energy requirements in sick preterm babies. 1994 [cited 2022 Apr 11];60–4. Available from: https://pubmed.ncbi.nlm.nih.gov/7734793/

33. Cooke RJ. Postnatal growth in preterm infants. Neonatal Nutr Metab Second Ed [Internet]. 2006 Jan 1 [cited 2022 Mar 8];47–57. Available from: https://www.cambridge.org/core/books/neonatal-nutrition-and-metabolism/postnatal-growth-in-preterm-infants/E1938202261F32B1EE4F751B3AFC38D1

34. Denne SC, Poindexter BB. Evidence supporting early nutritional support with parenteral amino acid infusion. Semin Perinatol [Internet]. 2007 Apr [cited 2022 Mar 8];31(2):56–60. Available from: https://pubmed.ncbi.nlm.nih.gov/17462489/

35. Lawrence PB. Breast milk. Best source of nutrition for term and preterm infants. Pediatr Clin North Am [Internet]. 1994 [cited 2022 Mar 15];41(5):925–41. Available from: https://pubmed.ncbi.nlm.nih.gov/7936781/

36. Jackson KM, Nazar AM. Breastfeeding, the immune response, and long-term health. J Am Osteopath Assoc. 2006 Apr;106(4):203–7.

37. Ballard O, Morrow AL. Human Milk Composition: Nutrients and Bioactive Factors. 2013 Feb 1 [cited 2022 Jun 6];60(1). Available from: /pmc/articles/PMC3586783/

38. A P. Regional Variations in the Composition of Human Milk. In: Jensen RC. Handbook of milk composition. 1995;919.

39. Castellote C, Casillas R, Ramírez-Santana C, Pérez-Cano FJ, Castell M, Moretones MG, et al. Premature delivery influences the immunological composition of colostrum and transitional and mature human milk. J Nutr [Internet]. 2011 Jun 1 [cited 2022 Jul 3];141(6):1181–7. Available from: https://pubmed.ncbi.nlm.nih.gov/21508211/

40. Weinberg ED. Human lactoferrin: a novel therapeutic with broad spectrum potential. J Pharm Pharmacol [Internet]. 2001 Oct 1 [cited 2022 Jul 3];53(10):1303–10. Available from: https://onlinelibrary.wiley.com/doi/full/10.1211/0022357011777792

41. Arslanoglu S, Boquien CY, King C, Lamireau D, Tonetto P, Barnett D, et al. Fortification of human milk for preterm infants: Update and recommendations of the European milk bank association (EMBA) working group on human milk fortification. Front Pediatr. 2019;7(MAR):76.

42. Lau C, Smith E, Schanler R. Coordination of suck-swallow and swallow respiration in preterm infants. Acta Pædiatrica [Internet]. 2003 Jun 1 [cited 2022 Mar 15];92(6):721–7. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1651-2227.2003.tb00607.

43. Sondheimer JM, Finkel, Yigael; Baker, Robert; Rosenthal, Philip; Sherman, Philip; Sondheimer J. A Critical Perspective on Trophic Feeding. J Pediatr Gastroenterol Nutr [Internet]. 2004 [cited 2022 Mar 31];38(3):237–8. Available from: https://journals.lww.com/jpgn/Fulltext/2004/03000/A_Critical_Perspective_on_Trophic_Feeding.1.aspx

44. Civardi E, Garofoli F, Tzialla C, Pozzi M, Stronati M. Trophic feeding for very preterm or very low birth weight infants. Ital J Pediatr 2015 411 [Internet]. 2015 Sep 24 [cited 2022 Apr 21];41(1):1–2. Available from: https://ijponline.biomedcentral.com/articles/10.1186/1824-7288-41-S1-A3

45. Morgan J, Bombell S, Mcguire W. Early trophic feeding versus enteral fasting for very preterm or very low birth weight infants. Cochrane Database Syst Rev [Internet]. 2013 Mar 28 [cited 2022 Mar 31];2013(3). Available from: https://www.cochranelibrary.com/cdsr/doi/10.1002/14651858.CD000504.pub4/full

46. Thakkar HS, Lakhoo K. Necrotizing Enterocolitis. Surg (United Kingdom) [Internet]. 2022 May 9 [cited 2022 Jun 12];37(11):628–31. Available from: https://www.ncbi.nlm.nih.gov/books/NBK513357/

47. Dudrick SJ, Palesty JA. Historical highlights of the development of total parenteral nutrition. Surg Clin North Am [Internet]. 2011 Jun [cited 2022 Mar 8];91(3):693–717. Available from: https://pubmed.ncbi.nlm.nih.gov/21621704/

48. Dudrick SJ, Malkan AD. The history, principles, and practice of parenteral nutrition in preterm neonates. Nutr Preterm Neonate A Clin Perspect. 2013 Nov 1;193–213.

49. Vinnars E, Wilmore D. Jonathan Roads Symposium Papers. History of parenteral nutrition. JPEN J Parenter Enteral Nutr [Internet]. 2003 [cited 2022 Mar 8];27(3):225–32. Available from: https://pubmed.ncbi.nlm.nih.gov/12757118/

50. Patel P, Bhatia J. Total parenteral nutrition for the very low birth weight infant. Semin Fetal Neonatal Med [Internet]. 2017 Feb 1 [cited 2021 Sep 21];22(1):2–7. Available from: https://pubmed.ncbi.nlm.nih.gov/27576106/

51. Pertkiewicz M, Dudrick SJ. Educational Paper Basics in clinical nutrition: Parenteral nutrition, ways of delivering parenteral nutrition and peripheral parenteral nutrition (PPN).

52. Payne-James JJ, Khawaja HT. First choice for total parenteral nutrition: The peripheral route. J Parenter Enter Nutr. 1993;17(5):468–78.

53. Rundjan L, Rohsiswatmo R, Paramita TN, Oeswadi CA. Closed Catheter Access System Implementation in Reducing the Bloodstream Infection Rate in Low Birth Weight Preterm Infants. Front Pediatr [Internet]. 2015 Mar 16 [cited 2022 Mar 8];3. Available from: /pmc/articles/PMC4360570/

54. O'Grady NP, Alexander M, Burns LA, Dellinger EP, Garland J, Heard SO, et al. Guidelines for the Prevention of Intravascular Catheter-related Infections. Clin Infect Dis An Off Publ Infect Dis Soc Am [Internet]. 2011 May [cited 2022 Mar 8];52(9):e162. Available from: /pmc/articles/PMC3106269/

55. Vlaardingerbroek H, van den Akker CHP, de Groof F, Hogewind-Schoonenboom JEE, Huang L, Riedijk MAA, et al. Amino Acids for the Neonate: Search for the Ideal Dietary Composition. Neoreviews [Internet]. 2011 Sep 1 [cited 2021 Sep 27];12(9):e506–16. Available from: https://neoreviews.aappublications.org/content/12/9/e506

56. Wu G. Amino acids: metabolism, functions, and nutrition. Amin Acids 2009 371 [Internet]. 2009 Mar 20 [cited 2022 Jun 26];37(1):1–17. Available from: https://link.springer.com/article/10.1007/s00726-009-0269-0

57. Wu G. Functional Amino Acids in Growth, Reproduction, and Health. Adv Nutr [Internet]. 2010 Nov 1 [cited 2021 Oct 4];1(1). Available from: https://academic.oup.com/advances/article/1/1/31/4591551

58. Iacone R, Scanzano C, Santarpia L, Cioffi I, Contaldo F, Pasanisi F. Macronutrients in Parenteral Nutrition: Amino Acids. Nutrients [Internet]. 2020 Mar 1 [cited 2022 Mar 8];12(3). Available from: /pmc/articles/PMC7146427/

59. Shelton CM, Clark AJ, Storm MC, Helms RA. Plasma Amino Acid Concentrations in 108 Children Receiving a Pediatric Amino Acid Formulation as Part of Parenteral Nutrition. J Pediatr Pharmacol Ther JPPT [Internet]. 2010 Apr 1 [cited 2022 Jul 3];15(2):110. Available from: /pmc/articles/PMC3018185/

60. Shah PS, Shah VS, Kelly LE. Arginine supplementation for prevention of necrotising enterocolitis in preterm infants. Cochrane Database Syst Rev [Internet]. 2017 Apr 11 [cited 2022 Jul 3];2017(4). Available from: /pmc/articles/PMC6478109/

61. Mark Feldman MD. In: Sleisenger and Fordtran's Gastrointestinal and Liver Disease Review and Assessment - 11th Edition [Internet]. [cited 2022 Jul 3]. Available from: https://www.elsevier.com/books/sleisenger-and-fordtran's-gastrointestinal-and-liver-disease-review-and-assessment/978-0-323-63659-9

62. Rivera A, Bell EF, Bier DM. Effect of intravenous amino acids on protein metabolism of preterm infants during the first three days of life. Pediatr Res [Internet]. 1993 [cited 2022 Apr 21];33(2):106–11. Available from: https://pubmed.ncbi.nlm.nih.gov/8433884/

63. Thureen PJ, Melara D, Fennessey P V., Hay WW. Effect of Low versus High Intravenous Amino Acid Intake on Very Low Birth Weight Infants in the Early Neonatal Period. Pediatr Res 2003 531 [Internet]. 2003 Jan 1 [cited 2022 Apr 21];53(1):24–32. Available from: https://www.nature.com/articles/pr20038

64. Ehrenkranz RA, Younes N, Lemons JA, Fanaroff AA, Donovan EF, Wright LL, et al. Longitudinal growth of hospitalized very low birth weight infants. Pediatrics [Internet]. 1999 Aug [cited 2022 Mar 17];104(2 Pt 1):280–9.

65. Bauer K, Bovermann G, Roithmaier A, Götz M, Prölss A, Versmold HT. Body composition, nutrition, and fluid balance during the first two weeks of life in preterm neonates weighing less than 1500 grams. J Pediatr [Internet]. 1991 [cited 2022 Jul 3];118(4 Pt 1):615–20. Available from: https://pubmed.ncbi.nlm.nih.gov/2007939/

66. Morgan C, Herwitker S, Badhawi I, Hart A, Tan M, Mayes K, et al. SCAMP: Standardised, concentrated, additional macronutrients, parenteral nutrition in very preterm infants: A phase IV randomised, controlled exploratory study of macronutrient intake, growth and other aspects of neonatal care. BMC Pediatr [Internet]. 2011 Jun 10 [cited 2022 Apr 11];11(1):1–11. Available from: https://bmcpediatr.biomedcentral.com/articles/10.1186/1471-2431-11-53

67. Cooke RWI, Lucas A, Yudkin PLN, Pryse-Davies J. Head circumference as an index of brain weight in the fetus and newborn. Early Hum Dev [Internet]. 1977 [cited 2022 Apr 26];1(2):145–9. Available from: https://pubmed.ncbi.nlm.nih.gov/617306/

68. Cheong JLY, Hunt RW, Anderson PJ, Howard K, Thompson DK, Wang HX, et al. Head growth in preterm infants: correlation with magnetic resonance imaging and neurodevelopmental outcome. Pediatrics [Internet]. 2008 Jun [cited 2022 Apr 11];121(6). Available from: https://pubmed.ncbi.nlm.nih.gov/18519457/

69. Hack M, Breslau N. Very Low Birth Weight Infants: Effects of Brain Growth During Infancy on Intelligence Quotient at 3 Years of Age. Pediatrics [Internet]. 1986 Feb 1 [cited 2022 Apr 11];77(2):196–202. Available from: /pediatrics/article/77/2/196/53821/Very-Low-Birth-Weight-Infants-Effects-of-Brain

70. Hack M, Breslau N, Weissman B, Aram D, Klein N, Borawski E. Effect of Very Low Birth Weight and Subnormal Head Size on Cognitive Abilities at School Age. http://dx.doi.org/101056/NEJM199107253250403 [Internet]. 2010 Jan 14 [cited 2022 Apr 11];325(4):231–7. Available from: https://www.nejm.org/doi/full/10.1056/nejm199107253250403

71. Cooke RWI, Foulder-Hughes L. Growth impairment in the very preterm and cognitive and motor performance at 7 years. Arch Dis Child [Internet]. 2003 Jun 1 [cited 2022 Apr 18];88(6):482–7. Available from: https://adc.bmj.com/content/88/6/482

72. Farooqi A, Hägglöf B, Sedin G, Gothefors L, Serenius F. Growth in 10- to 12-Year-Old Children Born at 23 to 25 Weeks' Gestation in the 1990s: A Swedish National Prospective Follow-up Study. Pediatrics [Internet]. 2006 Nov 1 [cited 2022 Apr 18];118(5):e1452–65. Available from: /pediatrics/article/118/5/e1452/69865/Growth-in-10-to-12-Year-Old-Children-Born-at-23-to

73. Ehrenkranz RA, Dusick AM, Vohr BR, Wright LL, Wrage LA, Poole WK. Growth in the Neonatal Intensive Care Unit Influences Neurodevelopmental and Growth Outcomes of Extremely Low Birth Weight Infants. Pediatrics [Internet]. 2006 Apr 1 [cited 2021 Oct 12];117(4):1253–61. Available from: https://pediatrics.aappublications.org/content/117/4/1253

74. Franz AR, Pohlandt F, Bode H, Mihatsch WA, Sander S, Kron M, et al. Intrauterine, early neonatal, and postdischarge growth and neurodevelopmental outcome at 5.4 years in extremely preterm infants after intensive neonatal nutritional support. Pediatrics [Internet]. 2009 Jan [cited 2022 Apr 11];123(1). Available from: https://pubmed.ncbi.nlm.nih.gov/19117831/

75. Ghods E, Kreissl A, Brandstetter S, Fuiko R, Widhalm K. Head circumference catch-up growth among preterm very low birth weight infants: effect on neurodevelopmental outcome. J Perinat Med [Internet]. 2011 Sep 1 [cited 2022 Apr 11];39(5):579–86. Available from: https://pubmed.ncbi.nlm.nih.gov/21740330/

76. South African Family Practice Nutrition and brain development Prof. Aida Mendoza-Salonga (Professor and Chair). 2007 [cited 2022 Apr 11]; Available from: https://www.tandfonline.com/action/journalInformation?journalCode=ojfp20

77. Isaacs EB, Gadian DG, Sabatini S, Chong WK, Quinn BT, Fischl BR, et al. The effect of early human diet on caudate volumes and IQ. Pediatr Res [Internet]. 2008 Mar [cited 2022 Jun 28];63(3):308–14. Available from: https://pubmed.ncbi.nlm.nih.gov/18287970/

78. Stephens BE, Walden R V., Gargus RA, Tucker R, McKinley L, Mance M, et al. First-Week Protein and Energy Intakes Are Associated With 18-Month Developmental Outcomes in Extremely Low Birth Weight Infants. Pediatrics [Internet]. 2009 May 1 [cited 2022 Jun 28];123(5):1337–43. Available from: /pediatrics/article/123/5/1337/71438/First-Week-Protein-and-Energy-Intakes-Are

79. Chan SHT, Johnson MJ, Leaf AA, Vollmer B, Chan S. NUTRITION AND NEURODEVELOPMENTAL OUTCOMES IN PRETERM INFANTS: A SYSTEMATIC REVIEW.

80. Lucas A, Morley R, Cole TJ. Randomised trial of early diet in preterm babies and later intelligence quotient. BMJ Br Med J [Internet]. 1998 Nov 11 [cited 2022 Jun 28];317(7171):1481. Available from: /pmc/articles/PMC28727/

81. Adams-Chapman I, Stoll BJ. Neonatal infection and long-term neurodevelopmental outcome in the preterm infant. Curr Opin Infect Dis [Internet]. 2006 Jun [cited 2022 Apr 18];19(3):290–7. Available from: https://journals.lww.com/co-infectiousdiseases/Fulltext/2006/06000/Neonatal_infection_and_long_term.12.aspx

82. Katz-Salamon M, Gerner EM, Jonsson B, Lagercrantz H. Early motor and mental development in very preterm infants with chronic lung disease. Arch Dis Child Fetal Neonatal Ed. 2000;83(1).

83. deRegnier RA, Roberts D, Ramsey D, Weaver RGJ, O'Shea TM. Association between the severity of chronic lung disease and first-year outcomes of very low birth weight infants. J Perinatol Off J Calif Perinat Assoc. 1997;17(5):375–82.

84. Ho MY, Yen YH, Hsieh MC, Chen HY, Chien SC, Hus-Lee SM. Early versus late nutrition support in premature neonates with respiratory distress syndrome. Nutrition [Internet]. 2003 Mar 1 [cited 2022 Apr 11];19(3):257–60.

85. Porcelli PJ, Sisk PM. Increased parenteral amino acid administration to extremely low-birth-weight infants during early postnatal life. J Pediatr Gastroenterol Nutr [Internet]. 2002 [cited 2022 Feb 28];34(2):174–9. Available from: https://pubmed.ncbi.nlm.nih.gov/11840036/

86. BB P, JC L, AM D, RA E. Early provision of parenteral amino acids in extremely low birth weight infants: relation to growth and neurodevelopmental outcome. J Pediatr [Internet]. 2006 [cited 2021 Oct 12];148(3). Available from: https://pubmed.ncbi.nlm.nih.gov/16615955/

87. Fomon SJ. Nutrition of normal infants. St. Louis: Mosby; 1993. 475 p.

88. Murray BM, Campos SP, Schoenl M, MacGillivray MH. Effect of dietary protein intake on renal growth: Possible role of insulin-like growth factor-I. J Lab Clin Med [Internet]. 1993 Dec 1 [cited 2022 Mar 14];122(6):677–85. Available from: http://www.translationalres.com/article/0022214393902503/fulltext

89. Jakobsson B, Aperia A. High protein intake accelerates the maturation of Na,K-ATPase in rat renal tubules. Acta Physiol Scand [Internet]. 1990 May 1 [cited 2022 Apr 21];139(1):1–7. Available from: https://europepmc.org/article/med/2162620

90. NICE. Neonatal Parenteral Nutrition. 2020;(February):2–5. Available from: www.nice.org.uk/guidance/ng154%0Ahttps://www.ucsfbenioffchildrens.org/pdf/manuals/47_TPN.pdf

91. Van Goudoever JB, Carnielli V, Darmaun D, Sainz de Pipaon M, Braegger C, Bronsky J, et al. ESPGHAN/ESPEN/ESPR/CSPEN guidelines on pediatric parenteral nutrition: Energy. Clin Nutr. 2018;37(6):2309–14.

92. Trivedi A, Sinn JKH. Early versus late administration of amino acids in preterm infants receiving parenteral nutrition. Cochrane database Syst Rev [Internet]. 2013 Jul 23 [cited 2022 Feb 28];2013(7). Available from: https://pubmed.ncbi.nlm.nih.gov/23881744/

93. Blanco CL, Gong AK, Green BK, Falck A, Schoolfield J, Liechty EA. Early changes in plasma amino acid concentrations during aggressive nutritional therapy in extremely low birth weight infants. J Pediatr [Internet]. 2011 [cited 2021 Nov 30];158(4). Available from: https://pubmed.ncbi.nlm.nih.gov/21129755/

94. Vlaardingerbroek H, Vermeulen MJ, Rook D, Van Den Akker CHP, Dorst K, Wattimena JL, et al. Safety and efficacy of early parenteral lipid and high-dose amino acid administration to very low birth weight infants. 2013 [cited 2022 Feb 28];163(3). Available from: https://pubmed.ncbi.nlm.nih.gov/23660378/

95. Leenders EKSM, De Waard M, Van Goudoever JB. Low- versus High-Dose and Early versus Late Parenteral Amino-Acid Administration in Very-Low-Birth-Weight Infants: A Systematic Review and Meta-Analysis. Neonatology [Internet]. 2018 Mar 1 [cited 2022 Feb 28];113(3):187–205. Available from: https://pubmed.ncbi.nlm.nih.gov/29268262/

96. Maggio L, Cota F, Gallini F, Lauriola V, Zecca C, Romagnoli C. Effects of high versus standard early protein intake on growth of extremely low birth weight infants. J Pediatr Gastroenterol Nutr [Internet]. 2007 Jan [cited 2022 Feb 28];44(1):124–9. Available from: https://pubmed.ncbi.nlm.nih.gov/17204965/

97. Morgan C, McGowan P, Herwitker S, Hart AE, Turner MA. Postnatal head growth in preterm infants: A randomized controlled parenteral nutrition study. Pediatrics [Internet]. 2014 [cited 2022 Feb 22];133(1). Available from: https://pubmed.ncbi.nlm.nih.gov/24379229/

98. Anderson TL, Muttart CR, Bieber MA, Nicholson JF, Heird WC. A controlled trial of glucose versus glucose and amino acids in premature infants. 1979 Jun 1 [cited 2022 Apr 26];94(6):947–51. Available from: https://pubmed.ncbi.nlm.nih.gov/109596/

99. RePub, Erasmus University Repository: Fetal Food - Preemie's Prerequisite? Studies on human fetal and neonatal protein metabolism [Internet]. [cited 2022 Apr 26]. Available from: https://repub.eur.nl/pub/20783/

100. HM I, MA J, RJ B, R D, RW K. Aggressive early total parental nutrition in low-birth-weight infants. J Perinatol [Internet]. 2004 Aug [cited 2021 Oct 12];24(8):482–6. Available from: https://pubmed.ncbi.nlm.nih.gov/15167885/

101. Wilson DC, Cairns P, Halliday HL, Reid M, McClure G, Dodge JA. Randomised controlled trial of an aggressive nutritional regimen in sick very low birthweight infants. Arch Dis Child Fetal Neonatal Ed [Internet]. 1997 [cited 2022 Mar 9];77(1):F4. Available from: /pmc/articles/PMC1720665/

102. Clark RH, Chace DH, Spitzer AR. Effects of Two Different Doses of Amino Acid Supplementation on Growth and Blood Amino Acid Levels in Premature Neonates Admitted to the Neonatal Intensive Care Unit: A Randomized, Controlled Trial. Pediatrics [Internet]. 2007 Dec 1 [cited 2021 Sep 27];120(6):1286–96. Available from: https://pediatrics.aappublications.org/content/120/6/1286

103. Tan M, Abernethy L, Cooke R. Improving head growth in preterm infants – a randomised controlled trial II: MRI and developmental outcomes in the first year. Arch Dis Child - Fetal Neonatal Ed [Internet]. 2008 Sep 1 [cited 2022 Apr 18];93(5):F342–6. Available from: https://fn.bmj.com/content/93/5/F342

104. Blanco CL, Gong AK, Schoolfield J, Green BK, Daniels W, Liechty EA, et al. Impact of early and high amino acid supplementation on ELBW infants at 2 years. J Pediatr Gastroenterol Nutr [Internet]. 2012 May [cited 2022 Mar 9];54(5):601–7. Available from: https://pubmed.ncbi.nlm.nih.gov/22228000/

105. Balasubramanian H, Nanavati RN, Kabra NS. Effect of two different doses of parenteral amino acid supplementation on postnatal growth of very low birth weight neonates, a randomized controlled trial. Indian Pediatr. 2013 Dec;50(12):1131–6.

106. Burattini I, Bellagamba MP, Spagnoli C, D'Ascenzo R, Mazzoni N, Peretti A, et al. Targeting 2.5 versus 4 g/kg/day of amino acids for extremely low birth weight infants: a randomized clinical trial. J Pediatr [Internet]. 2013 [cited 2022 Mar 9];163(5). Available from: https://pubmed.ncbi.nlm.nih.gov/23941670/

107. Scattolin S, Gaio P, Betto M, Palatron S, De Terlizzi F, Intini F, et al. Parenteral amino acid intakes: possible influences of higher intakes on growth and bone status in preterm infants. J Perinatol [Internet]. 2013 Jan [cited 2022 Mar 9];33(1):33–9. Available from: https://pubmed.ncbi.nlm.nih.gov/22517036/

108. Bellagamba MP, Carmenati E, D'Ascenzo R, Malatesta M, Spagnoli C, Biagetti C, et al. One Extra Gram of Protein to Preterm Infants From Birth to 1800 g: A Single-Blinded Randomized Clinical Trial. J Pediatr Gastroenterol Nutr [Internet]. 2016 Jun 1 [cited 2022 Mar 9];62(6):879–84. Available from: https://pubmed.ncbi.nlm.nih.gov/26418211/

109. Uthaya S, Liu X, Babalis D, Doré CJ, Warwick J, Bell J, et al. Nutritional Evaluation and Optimisation in Neonates: a randomized, double-blind controlled trial of amino acid regimen and intravenous lipid composition in preterm parenteral nutrition. Am J Clin Nutr [Internet]. 2016 Jun 1 [cited 2022 Feb 28];103(6):1443–52. Available from: https://pubmed.ncbi.nlm.nih.gov/27099248/

110. Balakrishnan M, Jennings A, Przystac L, Phornphutkul C, Tucker R, Vohr B, et al. Growth and Neurodevelopmental Outcomes of Early, High-Dose Parenteral Amino Acid Intake in Very Low Birth Weight Infants: A Randomized Controlled Trial. JPEN J Parenter Enteral Nutr [Internet]. 2018 Mar 1 [cited 2022 Mar 9];42(3):597–606. Available from: https://pubmed.ncbi.nlm.nih.gov/29187120/

111. Ogilvy-Stuart AL, Beardsall K. Management of hyperglycaemia in the preterm infant. Arch Dis Child Fetal Neonatal Ed [Internet]. 2010 Mar [cited 2022 Apr 21];95(2). Available from: https://pubmed.ncbi.nlm.nih.gov/20231218/

112. Morgan C. The potential risks and benefits of insulin treatment in hyperglycaemic preterm neonates. Early Hum Dev. 2015 Nov 1;91(11):655–9.

113. van Goudoever JB, Carnielli V, Darmaun D, Sainz de Pipaon M, Braegger C, Bronsky J, et al. ESPGHAN/ESPEN/ESPR/CSPEN guidelines on pediatric parenteral nutrition: Amino acids. Clin Nutr [Internet]. 2018 Dec 1 [cited 2022 Jun 14];37(6 Pt B):2315–23. Available from: https://pubmed.ncbi.nlm.nih.gov/30100107/

114. Mason DG, Puntis JWL, McCormick K, Smith N. Parenteral nutrition for neonates and children: a mixed bag. Arch Dis Child [Internet]. 2011 Mar 1 [cited 2022 Mar 9];96(3):209–10. Available from: https://adc.bmj.com/content/96/3/209

115. Alleway R, Begg S, Bull B, Blackman M, Freeth H, Jarman D, et al. A Mixed Bag An enquiry into the care of hospital patients receiving parenteral nutrition SUMMARY A Mixed Bag An enquiry into the care of hospital patients receiving parenteral nutrition A report by the National Confidential Enquiry into Patient Outcome an.

116. Ahmed M, Irwin S, Tuthill DP. Education and evidence are needed to improve neonatal parenteral nutrition practice. JPEN J Parenter Enteral Nutr [Internet]. 2004 [cited 2022 Mar 9];28(3):176–9. Available from: https://pubmed.ncbi.nlm.nih.gov/15141411/

117. Paize F, Mahaveer A, Morgan C. Effect of differences in parenteral nutrition policies on preterm macronutrient intake: a telephone survey of all UK level 3 neonatal services: Abstract G69 Table 1. Arch Dis Child. 2012 May;97(Suppl 1):A49.2-A50.

118. Grover A, Khashu M, Mukherjee A, Kairamkonda V. Iatrogenic malnutrition in neonatal intensive care units: urgent need to modify practice. JPEN J Parenter Enteral Nutr [Internet]. 2008 Mar [cited 2022 Mar 9];32(2):140–4. Available from: https://pubmed.ncbi.nlm.nih.gov/18407906/

119. Johnson JD, Albritton WL, Sunshine P. Hyperammonemia accompanying parenteral nutrition in newborn infants. J Pediatr. 1972 Jul 1;81(1):154–61.

120. Heird WC, Dell RB, Driscoll JM, Grebin B, Winters RW, WC H, et al. Metabolic acidosis resulting from intravenous alimentation mixtures containing synthetic amino acids. N Engl J Med [Internet]. 1972 Nov 9 [cited 2021 Sep 27];287(19):943–8. Available from: https://pubmed.ncbi.nlm.nih.gov/4627861/

121. Kotsopoulos K, Benadiba-Torch A, Cuddy A, Shah PS. Safety and efficacy of early amino acids in preterm <28 weeks gestation: prospective observational comparison. J Perinatol 2006 2612 [Internet]. 2006 Oct 5 [cited 2021 Sep 27];26(12):749–54. Available from: https://www.nature.com/articles/7211611

122. Te Braake FWJ, Van Den Akker CHP, Wattimena DJL, Huijmans JGM, Van Goudoever JB, FW te B, et al. Amino acid administration to premature infants directly after birth. J Pediatr [Internet]. 2005 Oct [cited 2021 Oct 12];147(4):457–61. Available from: https://pubmed.ncbi.nlm.nih.gov/16227030/

123. Thureen PJ, Anderson AH, Baron KA, Melara DL, Hay WW, Fennessey P V., et al. Protein balance in the first week of life in ventilated neonates receiving parenteral nutrition. Am J Clin Nutr [Internet]. 1998 [cited 2021 Oct 12];68(5):1128–35. Available from: https://pubmed.ncbi.nlm.nih.gov/9808233/

124. Morgan C, Burgess L. High Protein Intake Does Not Prevent Low Plasma Levels of Conditionally Essential Amino Acids in Very Preterm Infants Receiving Parenteral Nutrition. JPEN J Parenter Enteral Nutr [Internet]. 2017 Mar 1 [cited 2021 Nov 30];41(3):455–62. Available from: https://pubmed.ncbi.nlm.nih.gov/26150412/

125. Mayes K, Tan M, Morgan C. Effect of Hyperalimentation and Insulin-Treated Hyperglycemia on Tyrosine Levels in Very Preterm Infants Receiving Parenteral Nutrition. J Parenter Enter Nutr [Internet]. 2014 Jan 1 [cited 2021 Nov 19];38(1):92–8. Available from: https://onlinelibrary.wiley.com/doi/full/10.1177/0148607112467036

126. Poindexter BB, Ehrenkranz RA, Stoll BJ, Koch MA, Wright LL, Oh W, et al. Effect of parenteral glutamine supplementation on plasma amino acid concentrations in extremely low-birth-weight infants. Am J Clin Nutr. 2003;77(3):737–43.

127. Barker DJP, Osmond C, Simmonds SJ, Wield GA. The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life. BMJ Br Med J [Internet]. 1993 [cited 2022 Mar 9];306(6875):422. Available from: /pmc/articles/PMC1676496/?report=abstract

128. Barker DJP, Eriksson JG, Forsén T, Osmond C. Fetal origins of adult disease: strength of effects and biological basis. Int J Epidemiol [Internet]. 2002 Dec [cited 2022 Mar 9];31(6):1235–9. Available from: https://pubmed.ncbi.nlm.nih.gov/12540728/

129. Barker DJP. Adult consequences of fetal growth restriction. Clin Obstet Gynecol [Internet]. 2006 Jun [cited 2022 Mar 9];49(2):270–83. Available from: https://pubmed.ncbi.nlm.nih.gov/16721106/

130. Singer M, Deutschman CS, Seymour C, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA [Internet]. 2016 Feb 2 [cited 2022 Jul 3];315(8):801. Available from: /pmc/articles/PMC4968574/

131. Berrington JE, Hearn RI, Bythell M, Wright C, Embleton ND. Deaths in Preterm Infants: Changing Pathology Over 2 Decades. J Pediatr. 2012 Jan 1;160(1):49-53.e1.

132. Fleischmann C, Reichert F, Cassini A, Horner R, Harder T, Markwart R, et al. Global incidence and mortality of neonatal sepsis: a systematic review and meta-analysis. Arch Dis Child [Internet]. 2021 Aug 1 [cited 2022 Jul 3];106(8):745. Available from: /pmc/articles/PMC8311109/

133. Simonsen KA, Anderson-Berry AL, Delair SF, Dele Davies H. Early-Onset Neonatal Sepsis. Clin Microbiol Rev [Internet]. 2014 [cited 2022 Mar 8];27(1):21. Available from: /pmc/articles/PMC3910904/

134. Kim F, Polin RA, Hooven TA. Neonatal sepsis. BMJ [Internet]. 2020 Oct 1 [cited 2022 Mar 8];371:m3672. Available from: https://www.bmj.com/content/371/bmj.m3672

135. Dong Y, Speer CP. Late-onset neonatal sepsis: recent developments. Arch Dis Child Fetal Neonatal Ed [Internet]. 2015 May 1 [cited 2022 Mar 9];100(3):F257–63. Available from: https://pubmed.ncbi.nlm.nih.gov/25425653/

136. Vergnano S, Sharland M, Kazembe P, Mwansambo C, Heath PT. Neonatal sepsis: an international perspective. Arch Dis Child - Fetal Neonatal Ed [Internet]. 2005 May 1 [cited 2022 Mar 9];90(3):F220-FF224. Available from: https://fn.bmj.com/content/90/3/F220

137. Camacho-Gonzalez A, Spearman PW, Stoll BJ. Neonatal Infectious Diseases: Evaluation of Neonatal Sepsis. Pediatr Clin North Am [Internet]. 2013 Apr [cited 2022 Mar 8];60(2):367. Available from: /pmc/articles/PMC4405627/

138. Jan AI, Ramanathan R, Cayabyab RG. Chorioamnionitis and Management of Asymptomatic Infants ≥35 Weeks Without Empiric Antibiotics. Pediatrics [Internet]. 2017 Jul 1 [cited 2022 Mar 8];140(1).

139. Neonatal infection: antibiotics for prevention and treatment NICE guideline. 2021 [cited 2022 Mar 8]; Available from: www.nice.org.uk/guidance/ng195

140. Connell TG, Rele M, Cowley D, Buttery JP, Curtis N. How reliable is a negative blood culture result? Volume of blood submitted for culture in routine practice in a children's hospital. Pediatrics [Internet]. 2007 May [cited 2022 May 5];119(5):891–6. Available from: https://pubmed.ncbi.nlm.nih.gov/17473088/

141. Sharma AA, Jen R, Butler A, Lavoie PM. The developing human preterm neonatal immune system: A case for more research in this area. Clin Immunol. 2012 Oct 1;145(1):61–8.

142. Stevens JP, Eames M, Kent A, Halket S, Holt D, Harvey D. Long term outcome of neonatal meningitis. Arch Dis Child - Fetal Neonatal Ed [Internet]. 2003 May 1 [cited 2022 Jul 3];88(3):F179–84. Available from: https://fn.bmj.com/content/88/3/F179

143. Casaer MP, Mesotten D, Hermans G, Wouters PJ, Schetz M, Meyfroidt G, et al. Early versus Late Parenteral Nutrition in Critically Ill Adults. N Engl J Med [Internet]. 2011 Aug 11 [cited 2022 Jan 11];365(6):506–17. Available from: https://www.nejm.org/doi/full/10.1056/nejmoa1102662

144. Fivez T, Kerklaan D, Verbruggen S, Vanhorebeek I, Verstraete S, Tibboel D, et al. Impact of withholding early parenteral nutrition completing enteral nutrition in pediatric critically ill patients (PEPaNIC trial): Study protocol for a randomized controlled trial. Trials. 2015;16(1):1–9.

145. van Puffelen E, Vanhorebeek I, Joosten KFM, Wouters PJ, Van den Berghe G, Verbruggen SCAT. Early versus late parenteral nutrition in critically ill, term neonates: a preplanned secondary subgroup analysis of the PEPaNIC multicentre, randomised controlled trial. Lancet Child Adolesc Heal [Internet]. 2018;2(7):505–15. Available from: http://dx.doi.org/10.1016/S2352-4642(18)30131-7

146. Moltu SJ, Strømmen K, Blakstad EW, Almaas AN, Westerberg AC, Brække K, et al. Enhanced feeding in very-low-birth-weight infants may cause electrolyte disturbances and septicemia - A randomized, controlled trial. Clin Nutr [Internet]. 2013;32(2):207–12. Available from: http://dx.doi.org/10.1016/j.clnu.2012.09.004

147. Rocchi A, He C. Emerging roles of autophagy in metabolism and metabolic disorders. Front Biol (Beijing) [Internet]. 2015 Apr 1 [cited 2022 Jul 3];10(2):154. Available from: /pmc/articles/PMC4792296/

148. Deretic V, Saitoh T, Akira S. Autophagy in infection, inflammation and immunity. Nat Rev Immunol 2013 1310 [Internet]. 2013 Sep 25 [cited 2022 Feb 28];13(10):722–37. Available from: https://www.nature.com/articles/nri3532

149. Meijer AJ. Amino Acid Regulation of Autophagosome Formation. Methods Mol Biol [Internet]. 2008 [cited 2022 Jan 11];445:89–109. Available from: https://link.springer.com/protocol/10.1007/978-1-59745-157-4_5

150. Yorimitsu T, Klionsky DJ. Autophagy: molecular machinery for self-eating. 2005 Oct 25 [cited 2022 Jan 11];1542–52. Available from: https://pubmed.ncbi.nlm.nih.gov/16247502/

151. Klionsky DJ, Cregg JM, Dunn WA, Emr SD, Sakai Y, Sandoval I V., et al. A Unified Nomenclature for Yeast Autophagy-Related Genes. Dev Cell [Internet]. 2003 Oct 1 [cited 2022 Jan 11];5(4):539–45. Available from: http://www.cell.com/article/S153458070300296X/fulltext

152. Chang NC. Autophagy and Stem Cells: Self-Eating for Self-Renewal. Front Cell Dev Biol. 2020 Mar 4;8:138.

153. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol [Internet]. 2010 May [cited 2022 Mar 8];221(1):3–12. Available from: https://pubmed.ncbi.nlm.nih.gov/20225336/

154. Rabinowitz JD, White E. Autophagy and Metabolism. Science [Internet]. 2010 Dec 12 [cited 2022 Jun 25];330(6009):1344. Available from: /pmc/articles/PMC3010857/

155. Kanamori H, Takemura G, Maruyama R, Goto K, Tsujimoto A, Ogino A, et al. Functional Significance and Morphological Characterization of Starvation-Induced Autophagy in the Adult Heart. Am J Pathol [Internet]. 2009 [cited 2022 Jun 25];174(5):1705. Available from: /pmc/articles/PMC2671259/

156. Komatsu M, Waguri S, Koike M, Sou Y shin, Ueno T, Hara T, et al. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell [Internet]. 2007 Dec 14 [cited 2022 Jun 25];131(6):1149–63. Available from: https://pubmed.ncbi.nlm.nih.gov/18083104/

157. Cadwell K, Liu JY, Brown SL, Miyoshi H, Loh J, Lennerz JK, et al. A unique role for autophagy and Atg16L1 in Paneth cells in murine and human intestine. Nature [Internet]. 2008 Nov 11 [cited 2022 Jun 25];456(7219):259. Available from: /pmc/articles/PMC2695978/

158. Komatsu M, Waguri S, Chiba T, Murata S, Iwata JI, Tanida I, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature [Internet]. 2006 Jun 15 [cited 2022 Jun 25];441(7095):880–4. Available from: https://pubmed.ncbi.nlm.nih.gov/16625205/

159. Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, et al. The role of autophagy during the early neonatal starvation period. Nat 2004 4327020 [Internet]. 2004 Nov 3 [cited 2022 Jun 26];432(7020):1032–6. Available from: https://www.nature.com/articles/nature03029

160. Schiaffino S, Mammucari C, Sandri M. The role of autophagy in neonatal tissues: Just a response to amino acid starvation? 2008 [cited 2022 Jun 26]; Available from: https://www.tandfonline.com/action/journalInformation?journalCode=kaup20

161. I V, J G, S D, I D, M B, F G, et al. Insufficient activation of autophagy allows cellular damage to accumulate in critically ill patients. J Clin Endocrinol Metab [Internet]. 2011 Apr [cited 2021 Oct 12];96(4). Available from: https://pubmed.ncbi.nlm.nih.gov/21270330/

162. S D, I V, F G, I D, J G, B F, et al. Early parenteral nutrition evokes a phenotype of autophagy deficiency in liver and skeletal muscle of critically ill rabbits. Endocrinology [Internet]. 2012 May [cited 2021 Oct 12];153(5):2267–76. Available from: https://pubmed.ncbi.nlm.nih.gov/22396453/

163. Wu G, Jaeger LA, Bazer FW, Rhoads JM. Arginine deficiency in preterm infants: Biochemical mechanisms and nutritional implications. J Nutr Biochem. 2004 Aug 1;15(8):442–51.

164. Smith RJ, Wilmore DW. Glutamine nutrition and requirements. JPEN J Parenter Enteral Nutr [Internet]. 1990 [cited 2022 Feb 23];14(4 Suppl). Available from: https://pubmed.ncbi.nlm.nih.gov/2119461/

165. Columbus DA, Fiorotto ML, Davis TA. Leucine is a major regulator of muscle protein synthesis in neonates. Amino Acids [Internet]. 2015 [cited 2021 Dec 6];47(2):259. Available from: /pmc/articles/PMC4304911/

166. Escobar J, Frank JW, Suryawan A, Nguyen H V., Kimball SR, Jefferson LS, et al. Physiological rise in plasma leucine stimulates muscle protein synthesis in neonatal pigs by enhancing translation initiation factor activation. Am J Physiol Endocrinol Metab [Internet]. 2005 May [cited 2021 Dec 6];288(5). Available from: https://pubmed.ncbi.nlm.nih.gov/15644455/

167. Blanco C, McGill-Vargas L, Li C, Winter L, Nathanielsz P. High branched-chain amino acid concentrations are found in preterm baboons receiving intravenous amino acid solutions and mimic alterations found in preterm infants. JPEN J Parenter Enteral Nutr [Internet]. 2019 Nov 1 [cited 2022 Apr 26];43(8):1053. Available from: /pmc/articles/PMC7241650/

168. Rassin DK, Shattuck KE. Enteral amino acid and protein digestion, absorption, and metabolism. Neonatal Nutr Metab Second Ed [Internet]. 2006 Jan 1 [cited 2022 Sep 3];332–9. Available from: https://researchexperts.utmb.edu/en/publications/enteral-amino-acid-and-protein-digestion-absorption-and-metabolis

169. Drummond MJ, Rasmussen BB. Leucine-Enriched Nutrients and the Regulation of mTOR Signalling and Human Skeletal Muscle Protein Synthesis. Curr Opin Clin Nutr Metab Care [Internet]. 2008 May [cited 2021 Oct 12];11(3):222. Available from: /pmc/articles/PMC5096790/

170. Whitby T, McGowan P, Turner MA, Morgan C. Concentrated parenteral nutrition solutions and central venous catheter complications in preterm infants. Arch Dis Child Fetal Neonatal Ed. 2015;100(3):F250–2.

171. Jones IH, Hall NJ. Contemporary Outcomes for Infants with Necrotizing Enterocolitis-A Systematic Review. J Pediatr [Internet]. 2020 May 1 [cited 2022 Jun 12];220:86-92.e3. Available from: https://pubmed.ncbi.nlm.nih.gov/31982088/

172. Lee JS, Polin RA. Treatment and prevention of necrotizing enterocolitis. Semin Neonatol [Internet]. 2003 [cited 2022 Apr 25];8(6):449. Available from: /pmc/articles/PMC7128229/

173. Schnabl KL, Van Aerde JE, Thomson ABR, Clandinin MT. Necrotizing enterocolitis: A multifactorial disease with no cure. World J Gastroenterol [Internet]. 2008 Apr 14 [cited 2022 Apr 25];14(14):2142. Available from: /pmc/articles/PMC2703838/

174. Carling R, Moat S, Prunty H, Wright K, Powell A, Talbot R, et al. MULTICENTRE AGE-RELATED AMINO ACID REFERENCE INTERVALS FOR CEREBROSPINAL FLUID, PLASMA AND CSF:PLASMA RATIOS. J Inherit Metab Dis. 2011 Jan 1;34:S87–S87.

175. Law GK, Bertolo RF, Adjiri-Awere A, Pencharz PB, Ball RO. Adequate oral threonine is critical for mucin production and gut function in neonatal piglets. Am J Physiol Gastrointest Liver Physiol [Internet]. 2007 May [cited 2022 Jun 30];292(5). Available from: https://pubmed.ncbi.nlm.nih.gov/17234895/

176. Rigo J, Senterre J. Optimal threonine intake for preterm infants fed on oral or parenteral nutrition. JPEN J Parenter Enteral Nutr [Internet]. 1980 [cited 2022 Jun 30];4(1):15–7. Available from: https://pubmed.ncbi.nlm.nih.gov/6767045/

177. Boehm G, Cervantes H, Georgi G, Jelinek J, Sawatzki G, Wermuth B, et al. Effect of increasing dietary threonine intakes on amino acid metabolism of the central nervous system and peripheral tissues in growing rats. Pediatr Res [Internet]. 1998 [cited 2022 Jun 30];44(6):900–6. Available from: https://pubmed.ncbi.nlm.nih.gov/9853925/

178. Bertolo RFP, Chen CZL, Law G, Pencharz PB, Ball RO. Threonine requirement of neonatal piglets receiving total parenteral nutrition is considerably lower than that of piglets receiving an identical diet intragastrically. J Nutr [Internet]. 1998 [cited 2022 Jun 30];128(10):1752–9. Available from: https://pubmed.ncbi.nlm.nih.gov/9772146/

179. Van Der Schoor SRD, Wattimena DL, Huijmans J, Vermes A, Van Goudoever JB. The gut takes nearly all: Threonine kinetics in infants. Am J Clin Nutr. 2007 Oct 1;86(4):1132–8.

180. Chapman KP, Courtney-Martin G, Moore AM, Ball RO, Pencharz PB. Threonine requirement of parenterally fed postsurgical human neonates. Am J Clin Nutr [Internet]. 2009 Jan 1 [cited 2022 Jun 30];89(1):134–41. Available from: https://academic.oup.com/ajcn/article/89/1/134/4598231

181. Rand KM, Austin NC, Inder TE, Bora S, Woodward LJ. Neonatal Infection and Later Neurodevelopmental Risk in the Very Preterm Infant. J Pediatr [Internet]. 2016 Mar 1 [cited 2022 Jun 8];170:97–104. Available from: http://www.jpeds.com/article/S0022347615013700/fulltext

182. Calder PC. Immunonutrition: May have beneficial effects in surgical patients. BMJ Br Med J [Internet]. 2003 Jul 19 [cited 2022 May 12];327(7407):117. Available from: /pmc/articles/PMC1126497/

183. Watford M. Glutamine Metabolism and Function in Relation to Proline Synthesis and the Safety of Glutamine and Proline Supplementation. J Nutr [Internet]. 2008 Oct 1 [cited 2022 Jun 8];138(10):2003S-2007S. Available from: https://academic.oup.com/jn/article/138/10/2003S/4670096

184. Manso HECCC, Filho HCM, de Carvalho LE, Kutschenko M, Nogueira ET, Watford M. Glutamine and glutamate supplementation raise milk glutamine concentrations in lactating gilts. J Anim Sci Biotechnol [Internet]. 2012 Feb [cited 2022 Jun 8];3(1):2. Available from: /pmc/articles/PMC3415122/

185. Rombeau JL. A review of the effects of glutamine-enriched diets on experimentally induced enterocolitis. JPEN J Parenter Enteral Nutr. 1990;14(4 Suppl).

186. Tao KM, Li XQ, Yang LQ, Yu WF, Lu ZJ, Sun YM, et al. Glutamine supplementation for critically ill adults. Cochrane database Syst Rev [Internet]. 2014 Sep 9 [cited 2022 Feb 23];2014(9). Available from: https://pubmed.ncbi.nlm.nih.gov/25199493/

187. Novak F, Heyland DK, Avenell A, Drover JW, Su X. Glutamine supplementation in serious illness: a systematic review of the evidence. Crit Care Med [Internet]. 2002 Sep 1 [cited 2022 Feb 23];30(9):2022–9. Available from: https://pubmed.ncbi.nlm.nih.gov/12352035/

188. Moe-Byrne T, Brown JVE, Mcguire W. Glutamine supplementation to prevent morbidity and mortality in preterm infants. Cochrane Database Syst Rev [Internet]. 2016 Jan 12 [cited 2022 Feb 23];2016(1). Available from: https://pubmed.ncbi.nlm.nih.gov/27089158/

189. Poindexter BB, Ehrenkranz RA, Stoll BJ, Wright LL, Poole WK, Oh W, et al. Parenteral Glutamine Supplementation Does Not Reduce the Risk of Mortality or Late-Onset Sepsis in Extremely Low Birth Weight Infants. Pediatrics [Internet]. 2004 May 1 [cited 2022 Jun 8];113(5):1209–15. Available from: /pediatrics/article/113/5/1209/66687/Parenteral-Glutamine-Supplementation-Does-Not

190. Wu G, Knabe DA. Arginine synthesis in enterocytes of neonatal pigs. Am J Physiol [Internet]. 1995 [cited 2022 Jun 27];269(3 Pt 2). Available from: https://pubmed.ncbi.nlm.nih.gov/7573565/

191. te Braake FWJ, van den Akker CHP, Riedijk MA, van Goudoever JB. Parenteral amino acid and energy administration to premature infants in early life. Semin Fetal Neonatal Med. 2007 Feb 1;12(1):11–8.

192. Heird WC, Nicholson JF, Driscoll JM, Schullinger JN, Winters RW. Hyperammonemia resulting from intravenous alimentation using a mixture of synthetic L-amino acids: A preliminary report. J Pediatr. 1972;81(1):162–5.

193. Wu G, Bazer FW, Davis TA, Kim SW, Li P, Marc Rhoads J, et al. Arginine metabolism and nutrition in growth, health and disease. Amino Acids [Internet]. 2009 May [cited 2022 Feb 23];37(1):153–68. Available from: https://pubmed.ncbi.nlm.nih.gov/19030957/

194. Weiss SL, Haymond S, Ranaivo HR, Wang D, De Jesus VR, Chace DH, et al. Evaluation of Asymmetric Dimethylarginine, Arginine, and Carnitine Metabolism in Pediatric Sepsis. Pediatr Crit Care Med [Internet]. 2012 Jul [cited 2022 Jun 8];13(4):e210. Available from: /pmc/articles/PMC3392424/

195. Freund H, Atamian S, Holroyde J, Fischer JE. Plasma amino acids as predictors of the severity and outcome of sepsis. Ann Surg [Internet]. 1979 [cited 2022 Jun 8];190(5):571–6. Available from: https://pubmed.ncbi.nlm.nih.gov/389183/

196. Badurdeen S, Mulongo M, Berkley JA. Arginine depletion increases susceptibility to serious infections in preterm newborns. Pediatr Res [Internet]. 2015 Feb 11 [cited 2022 Feb 23];77(2):290–7. Available from: https://pubmed.ncbi.nlm.nih.gov/25360828/

197. Munder M, Schneider H, Luckner C, Giese T, Langhans CD, Fuentes JM, et al. Suppression of T-cell functions by human granulocyte arginase. Blood [Internet]. 2006 Sep 1 [cited 2022 Jun 8];108(5):1627–34. Available from: https://pubmed.ncbi.nlm.nih.gov/16709924/

198. Polycarpou E, Zachaki S, Tsolia M, Papaevangelou V, Polycarpou N, Briana DD, et al. Enteral L-arginine supplementation for prevention of necrotizing enterocolitis in very low birth weight neonates: a double-blind randomized pilot study of efficacy and safety. JPEN J Parenter Enteral Nutr [Internet]. 2013 Sep [cited 2022 May 5];37(5):617–22. Available from: https://pubmed.ncbi.nlm.nih.gov/23329787/

199. Zamora SA, Amin HJ, McMillan DD, Kubes P, Fick GH, Butzner JD, et al. Plasma l -arginine concentrations in premature infants with necrotizing enterocolitis. J Pediatr [Internet]. 1997 Aug 1 [cited 2022 Feb 23];131(2):226–32. Available from: http://www.jpeds.com/article/S0022347697701586/fulltext

200. Amin HJ, Zamora SA, McMillan DD, Fick GH, Butzner JD, Parsons HG, et al. Arginine supplementation prevents necrotizing enterocolitis in the premature infant. J Pediatr [Internet]. 2002 [cited 2022 Feb 23];140(4):425–31. Available from: https://pubmed.ncbi.nlm.nih.gov/12006956/

201. McCaffrey MJ, Bose CL, Reite PD, Stiles AD. Effect of L-arginine infusion on infants with persistent pulmonary hypertension of the newborn. Biol Neonate [Internet]. 1995 [cited 2022 Feb 23];67(4):240–3. Available from: https://pubmed.ncbi.nlm.nih.gov/7647147/

202. Vosatka RJ, Kashyap S, Trifiletti RR. Arginine Deficiency Accompanies Persistent Pulmonary Hypertension of the Newborn. Neonatology [Internet]. 1994 [cited 2022 Jun 8];66(2–3):65–70. Available from: https://www.karger.com/Article/FullText/244091

203. Butler SO, Btaiche IF, Alaniz C. Relationship between hyperglycemia and infection in critically ill patients. Pharmacotherapy [Internet]. 2005 Jul [cited 2022 Jun 8];25(7):963–76. Available from: https://pubmed.ncbi.nlm.nih.gov/16006275/

204. Brocklehurst P, Brearley S, Haque K, Leslie A, Salt A, Stenson B, et al. The INIS Study. International Neonatal Immunotherapy Study: non-specific intravenous immunoglobulin therapy for suspected or proven neonatal sepsis: an international, placebo controlled, multicentre randomised trial. BMC Pregnancy Childbirth [Internet]. 2008 Dec 8 [cited 2022 Jun 27];8:52. Available from: /pmc/articles/PMC2626572/

205. Henderickx JGE, Zwittink RD, Renes IB, van Lingen RA, van Zoeren-Grobben D, Jebbink LJG, et al. Maturation of the preterm gastrointestinal tract can be defined by host and microbial markers for digestion and barrier defense. Sci Rep [Internet]. 2021 Dec 1 [cited 2022 Jun 24];11(1):12808. Available from: /pmc/articles/PMC8211855/

206. Miller JE, Hammond GC, Strunk T, Moore HC, Leonard H, Carter KW, et al. Association of gestational age and growth measures at birth with infection-related admissions to hospital throughout childhood: a population-based, data-linkage study from Western Australia. Lancet Infect Dis. 2016 Aug 1;16(8):952–61.

207. Cynober LA. Plasma amino acid levels with a note on membrane transport: characteristics, regulation, and metabolic significance. Nutrition. 2002 Sep 1;18(9):761–6.

208. Van Goudoever JB. Amino acid metabolism and protein accretion. Neonatal Nutr Metab Second Ed. 2006;115–21.

209. Iroh Tam PY, Bendel CM. Diagnostics for neonatal sepsis: current approaches and future directions. Pediatr Res 2017 824 [Internet]. 2017 Jun 28 [cited 2022 May 5];82(4):574–83. Available from: https://www.nature.com/articles/pr2017134

210. Lamy B, Dargère S, Arendrup MC, Parienti JJ, Tattevin P. How to Optimize the Use of Blood Cultures for the Diagnosis of Bloodstream Infections? A State-of-the Art. Front Microbiol [Internet]. 2016 [cited 2022 Jun 8];7(MAY):697. Available from: /pmc/articles/PMC4863885/

211. Wynn JL, Polin RA. Progress in the management of neonatal sepsis: the importance of a consensus definition. Pediatr Res 2018 831 [Internet]. 2017 Oct 11 [cited 2022 May 5];83(1):13–5. Available from: https://www.nature.com/articles/pr2017224

212. Mishra UK, Jacobs SE, Doyle LW, Garland SM. Newer approaches to the diagnosis of early onset neonatal sepsis. Arch Dis Child Fetal Neonatal Ed [Internet]. 2006 May [cited 2022 May 10];91(3). Available from: https://pubmed.ncbi.nlm.nih.gov/16632649/

213. PRISMA-P (Preferred Reporting Items for Systematic review and Meta-Analysis Protocols) 2015 checklist: recommended items to address in a systematic review protocol* Section and topic Item No Checklist item.

214. Huggins J, Thomas J, Chandler J, Cumpston M, Li T. Cochrane Handbook for Systematic Reviews of Interventions | Cochrane Training [Internet]. Version 6.2. 2021 [cited 2021 Oct 11]. Available from: https://training.cochrane.org/handbook/current

215. Premakumar CM, Turner MA, Morgan C. Relationship between arginine intake in parenteral nutrition and preterm neonatal population plasma arginine concentrations: A systematic review. Nutr Rev. 2019;77(12):878–89.

216. Ng PC, Brownlee KG, Kelly EJ, Henderson MJ, Smith M, Dear PRF. Changes in the plasma aminogram of parenterally fed infants treated with dexamethasone for bronchopulmonary dysplasia. Arch Dis Child [Internet]. 1992 [cited 2021 Nov 30];67(10 Spec No):1193–5. Available from: https://pubmed.ncbi.nlm.nih.gov/1444557/

217. Bulbul A, Okan F, Bulbul L, Nuhoglu A. Effect of low versus high early parenteral nutrition on plasma amino acid profiles in very low birth-weight infants. J Matern Fetal Neonatal Med [Internet]. 2012 Jun [cited 2021 Nov 30];25(6):770–6. Available from: https://pubmed.ncbi.nlm.nih.gov/21770835/

218. Chessex P, Zebiche H, Pineault M, Lepage D, Dallaire L. Effect of amino acid composition of parenteral solutions on nitrogen retention and metabolic response in very-low-birth weight infants. J Pediatr [Internet]. 1985 [cited 2021 Nov 30];106(1):111–7. Available from: https://pubmed.ncbi.nlm.nih.gov/3917496/

219. Kalhan SC, Parimi PS, Gruca LL, Hanson RW. Glutamine supplement with parenteral nutrition decreases whole body proteolysis in low birth weight infants. J Pediatr [Internet]. 2005 [cited 2021 Nov 30];146(5):642–7. Available from: https://pubmed.ncbi.nlm.nih.gov/15870668/

220. Mitton SG, Burston D, Brueton MJ, Kovar IZ. Plasma amino acid profiles in preterm infants receiving Vamin 9 glucose or Vamin infant. Early Hum Dev [Internet]. 1993 [cited 2021 Nov 30];32(1):71–8. Available from: https://pubmed.ncbi.nlm.nih.gov/8462435/

221. Ogata ES, Boehm JJ, Deddish RB. Clinical trial of a 6.5% amino acid infusion in appropriate-for-gestational-age premature neonates. Acta Chir Scand. 1983;149(SUPPL. 517):39–48.

222. Thornton L, Griffin E. Evaluation of a taurine containing amino acid solution in parenteral nutrition. Arch Dis Child [Internet]. 1991 [cited 2021 Nov 30];66(1 Spec No):21–5. Available from: https://pubmed.ncbi.nlm.nih.gov/1899989/

223. Van Goudoever JB, Sulkers EJ, Timmerman M, Carnielli VP, Sauer PJJ, Huijmans JGM, et al. Amino acid solutions for premature neonates during the first week of life: the role of N-acetyl-L-cysteine and N-acetyl-L-tyrosine. JPEN J Parenter Enteral Nutr [Internet]. 1994 [cited 2021 Nov 30];18(5):404–8. Available from: https://pubmed.ncbi.nlm.nih.gov/7815670/

224. Oglesbee D, Sanders KA, Lacey JM, Magera MJ, Casetta B, Strauss KA, et al. Second-Tier Test for Quantification of Alloisoleucine and Branched-Chain Amino Acids in Dried Blood Spots to Improve Newborn Screening for Maple Syrup Urine Disease (MSUD). Clin Chem [Internet]. 2008 Mar 1 [cited 2022 Jan 20];54(3):542–9. Available from: https://academic.oup.com/clinchem/article/54/3/542/5628443

225. Van Vliet K, Van Ginkel WG, Van Dam E, De Blaauw P, Koehorst M, Kingma HA, et al. Dried blood spot versus venous blood sampling for phenylalanine and tyrosine. Orphanet J Rare Dis [Internet]. 2020 Apr 3 [cited 2022 Jan 20];15(1):1–8. Available from: https://ojrd.biomedcentral.com/articles/10.1186/s13023-020-1343-7

226. PY W, N E, MC S. Plasma amino acid pattern in normal term breast-fed infants. J Pediatr [Internet]. 1986 [cited 2021 Oct 13];109(2):347–9. Available from: https://pubmed.ncbi.nlm.nih.gov/3734973/

227. Scott PH, Sandham S, Balmer SE, Wharton BA. Diet-related reference values for plasma amino acids in newborns measured by reversed-phase HPLC. Clin Chem [Internet]. 1990 Nov 1 [cited 2021 Nov 25];36(11):1922–7. Available from: https://academic.oup.com/clinchem/article/36/11/1922/5648833

228. S K, KF S, M F, RB D, R R, WC H. Growth, nutrient retention, and metabolic response of low-birth-weight infants fed supplemented and unsupplemented preterm human milk. Am J Clin Nutr [Internet]. 1990 [cited 2021 Oct 13];52(2):254–62. Available from: https://pubmed.ncbi.nlm.nih.gov/2375291/

229. Pittard WB, Geddes KM, Picone TA. Cord Blood Amino Acid Concentrations from Neonates of 23—41 Weeks Gestational Age. J Parenter Enter Nutr. 1988;12(2):167–9.

230. ROBIS tool | Bristol Medical School: Population Health Sciences | University of Bristol [Internet]. [cited 2022 Sep 2]. Available from: https://www.bristol.ac.uk/population-health-sciences/projects/robis/robis-tool/

231. QUADAS-2 | Bristol Medical School: Population Health Sciences | University of Bristol [Internet]. [cited 2022 Sep 2]. Available from: https://www.bristol.ac.uk/population-health-sciences/projects/quadas/quadas-2/

232. Damhuis SE, Bloomfield FH, Khalil A, Daly M, Ganzevoort W, Gordijn SJ. A Core Outcome Set and minimum reporting set for intervention studies in growth restriction in the NEwbOrN: the COSNEON study. Pediatr Res 2020 896 [Internet]. 2020 Sep 14 [cited 2022 Sep 2];89(6):1380–5. Available from: https://www.nature.com/articles/s41390-020-01119-5

233. Webbe JWH, Duffy JMN, Afonso E, Al-Muzaffar I, Brunton G, Greenough A, et al. Core outcomes in neonatology: development of a core outcome set for neonatal research. Arch Dis Child - Fetal Neonatal Ed [Internet]. 2020 Jul 1 [cited 2022 Sep 2];105(4):425–31. Available from: https://fn.bmj.com/content/105/4/425

234. Burgess L, Morgan C, Mayes K, Tan M. Plasma arginine levels and blood glucose control in very preterm infants receiving 2 different parenteral nutrition regimens. JPEN J Parenter Enteral Nutr [Internet]. 2014 Feb [cited 2021 Nov 23];38(2):243–53. Available from: https://pubmed.ncbi.nlm.nih.gov/23474648/

235. Pineault M, Chessex P, Lepage D, Dallaire L, Brisson G, Qureshi I. Total parenteral nutrition in very low birth weight infants with Travasol 10% blend C. JPEN J Parenter Enteral Nutr [Internet]. 1986 [cited 2021 Nov 30];10(3):296–9. Available from: https://pubmed.ncbi.nlm.nih.gov/3086589/

236. Sterne JAC, Savović J, Page MJ, Elbers RG, Blencowe NS, Boutron I, et al. RoB 2: a revised tool for assessing risk of bias in randomised trials. BMJ [Internet]. 2019 Aug 28 [cited 2022 Jan 20];366. Available from: https://www.bmj.com/content/366/bmj.l4898

237. Higgins JPT, Altman DG, Gøtzsche PC, Jüni P, Moher D, Oxman AD, et al. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. BMJ [Internet]. 2011 Oct 18 [cited 2022 Jan 20];343(7829). Available from: https://www.bmj.com/content/343/bmj.d5928

238. What is GRADE? | BMJ Best Practice [Internet]. [cited 2022 Jan 21]. Available from: https://bestpractice.bmj.com/info/toolkit/learn-ebm/what-is-grade/

239. Carling RS, McDonald BAC, Austin D, Burden D, Correia J, Leung J, et al. Challenging the status quo: A comparison of ion exchange chromatography with liquid chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry methods for the measurement of amino acids in human plasma. Ann Clin Biochem [Internet]. 2020 Jul 1 [cited 2021 Dec 2];57(4):277–90. Available from: https://pubmed.ncbi.nlm.nih.gov/32438818/

240. Samuel TM, Zhou Q, Giuffrida F, Munblit D, Verhasselt V, Thakkar SK. Nutritional and Non-nutritional Composition of Human Milk Is Modulated by Maternal, Infant, and Methodological Factors. Front Nutr [Internet]. 2020 Sep 16 [cited 2022 Jul 3];7:576133. Available from: /pmc/articles/PMC7557356/

241. Critical Appraisal Skills Programme (2019). CASP Randomised Control Trial checklist.

242. Neu J. Gastrointestinal maturation and implications for infant feeding. Early Hum Dev [Internet]. 2007 Dec [cited 2022 Jan 21];83(12):767–75. Available from: https://pubmed.ncbi.nlm.nih.gov/17913404/

243. Wan X, Wang W, Liu J, Tong T. Estimating the sample mean and standard deviation from the sample size, median, range and/or interquartile range. BMC Med Res Methodol [Internet]. 2014 Dec 1 [cited 2022 Sep 3];14(1):1–13. Available from: https://bmcmedresmethodol.biomedcentral.com/articles/10.1186/1471-2288-14-135

244. Steiner L, Diesner SC, Voitl P. Risk of infection in the first year of life in preterm children: An Austrian observational study. PLoS One [Internet]. 2019 Dec 1 [cited 2022 Jul 2];14(12). Available from: /pmc/articles/PMC6901347/

245. Ygberg S, Nilsson A. The developing immune system – from foetus to toddler. Acta Paediatr [Internet]. 2012 Feb 1 [cited 2022 May 10];101(2):120–7. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1651-2227.2011.02494.x

246. Ledford H. Fetal immune system revs up in the womb. Nature. 2017 Jun 14;546(7658):335–6.

247. Lawn JE, Cousens S, Zupan J. 4 Million neonatal deaths: When? Where? Why? Lancet. 2005 Mar 5;365(9462):891–900.

248. Kumar SKM, Bhat BV. Distinct mechanisms of the newborn innate immunity. Immunol Lett. 2016 May 1;173:42–54.

249. Neonatal Immunology | British Society for Immunology [Internet]. [cited 2022 Jun 6]. Available from: https://www.immunology.org/public-information/bitesized-immunology/immune-development/neonatal-immunology

250. van den Berg JP, Westerbeek EAM, van der Klis FRM, Berbers GAM, Van Elburg RM. Transplacental transport of IgG antibodies to preterm infants: a review of the literature. Early Hum Dev [Internet]. 2011 Feb [cited 2022 Mar 8];87(2):67–72. Available from: https://pubmed.ncbi.nlm.nih.gov/21123010/

251. Field CJ. The immunological components of human milk and their effect on immune development in infants. J Nutr [Internet]. 2005 [cited 2022 Jul 3];135(1):1–4. Available from: https://pubmed.ncbi.nlm.nih.gov/15623823/

252. Belkaid Y, Hand TW. Role of the Microbiota in Immunity and inflammation. Cell [Internet]. 2014 Mar 3 [cited 2022 Jun 3];157(1):121. Available from: /pmc/articles/PMC4056765/

253. Relman DA. Maternal IgA: Matchmaking in Early Childhood. Immunity. 2019 Aug 20;51(2):211–3.

254. Salzman NH. The role of the microbiome in immune cell development. Ann Allergy, Asthma Immunol. 2014 Dec 1;113(6):593–8.

255. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005 114 [Internet]. 2005 Apr 5 [cited 2022 Jun 6];11(4):S45–53. Available from: https://www.nature.com/articles/nm1213

256. Chirico G. Immune development in late preterm neonates. Ital J Pediatr 2014 402 [Internet]. 2014 Oct 9 [cited 2022 May 10];40(2):1–1. Available from: https://ijponline.biomedcentral.com/articles/10.1186/1824-7288-40-S2-A40

257. Urlichs F, Speer CP. Neutrophil Function in Preterm and Term Infants. Neoreviews [Internet]. 2004 Oct 1 [cited 2022 Jun 9];5(10):e417–30. Available from: /neoreviews/article/5/10/e417/88544/Neutrophil-Function-in-Preterm-and-Term-Infants

258. Filias A, Theodorou GL, Mouzopoulou S, Varvarigou AA, Mantagos S, Karakantza M. Phagocytic ability of neutrophils and monocytes in neonates. BMC Pediatr [Internet]. 2011 Apr 14 [cited 2022 Jun 9];11(1):1–6. Available from: https://bmcpediatr.biomedcentral.com/articles/10.1186/1471-2431-11-29

259. Mahdi M, Maródi L. Monocytes in Neonatal Immunity. Neoreviews [Internet]. 2010 Oct 1 [cited 2022 Jun 9];11(10):e558–65. Available from: /neoreviews/article/11/10/e558/88344/Monocytes-in-Neonatal-Immunity

260. Winterberg T, Vieten G, Meier T, Yu Y, Busse M, Hennig C, et al. Distinct phenotypic features of neonatal murine macrophages. Eur J Immunol [Internet]. 2015 Jan 1 [cited 2022 Jun 9];45(1):214–24. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/eji.201444468

261. Lee YC, Lin SJ. Neonatal Natural Killer Cell Function: Relevance to Antiviral Immune Defense. Clin Dev Immunol [Internet]. 2013 [cited 2022 Jun 9];2013. Available from: /pmc/articles/PMC3770027/

262. Papaioannou NE, Pasztoi M, Schraml BU. Understanding the Functional Properties of Neonatal Dendritic Cells: A Doorway to Enhance Vaccine Effectiveness? Front Immunol [Internet]. 2018 [cited 2022 Jun 9];9(JAN):3123. Available from: /pmc/articles/PMC6335269/

263. Korir ML, Manning SD, Davies HD. Intrinsic Maturational Neonatal Immune Deficiencies and Susceptibility to Group B Streptococcus Infection. Clin Microbiol Rev [Internet]. 2017 Oct 1 [cited 2022 Jun 6];30(4):973. Available from: /pmc/articles/PMC5608877/

264. McGreal EP, Hearne K, Spiller OB. Off to a slow start: under-development of the complement system in term newborns is more substantial following premature birth. Immunobiology [Internet]. 2012 Feb [cited 2022 Jun 6];217(2):176–86. Available from: https://pubmed.ncbi.nlm.nih.gov/21868122/

265. Walker JC, Smolders MAJC, Gemen EFA, Antonius TAJ, Leuvenink J, De Vries E. Development of Lymphocyte Subpopulations in Preterm Infants. Scand J Immunol [Internet]. 2011 Jan 1 [cited 2022 Jun 6];73(1):53–8. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1365-3083.2010.02473.x

266. Zaghouani H, Hoeman CM, Adkins B. Neonatal immunity: faulty T-helpers and the shortcomings of dendritic cells. Trends Immunol [Internet]. 2009 Dec [cited 2022 Jun 6];30(12):585. Available from: /pmc/articles/PMC2787701/

267. Belderbos M, Levy O, Bont L. Neonatal innate immunity in allergy development. Curr Opin Pediatr [Internet]. 2009 Dec [cited 2022 Jun 6];21(6):762–9. Available from: https://journals.lww.com/co-pediatrics/Fulltext/2009/12000/Neonatal_innate_immunity_in_allergy_development.11.aspx

268. Klein Klouwenberg P, Bont L. Neonatal and Infantile Immune Responses to Encapsulated Bacteria and Conjugate Vaccines. Clin Dev Immunol [Internet]. 2008 [cited 2022 Jun 6];2008. Available from: /pmc/articles/PMC2553187/

269. Hornef MW, Torow N. 'Layered immunity' and the 'neonatal window of opportunity' – timed succession of non-redundant phases to establish mucosal host–microbial homeostasis after birth. Immunology [Internet]. 2020 Jan 1 [cited 2022 Jun 6];159(1):15–25. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/imm.13149

270. Valdor R, MacIan F. Autophagy and the Regulation of the Immune Response. Pharmacol Res [Internet]. 2012 Dec [cited 2022 Mar 8];66(6):475. Available from: /pmc/articles/PMC3508673/

271. Oh JE, Lee HK. Pattern recognition receptors and autophagy. Front Immunol. 2014;5(JUN):300.

272. Nakahira K, Haspel JA, Rathinam VAK, Lee SJ, Dolinay T, Lam HC, et al. Autophagy proteins regulate innate immune response by inhibiting NALP3 inflammasome-mediated mitochondrial DNA release. Nat Immunol [Internet]. 2011 Mar [cited 2022 Jan 11];12(3):222. Available from: /pmc/articles/PMC3079381/

273. Dengjel J, Schoor O, Fischer R, Reich M, Kraus M, Müller M, et al. Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. Proc Natl Acad Sci U S A [Internet]. 2005 May 31 [cited 2022 Mar 8];102(22):7922–7. Available from: https://pubmed.ncbi.nlm.nih.gov/15894616/

274. Pua HH, Dzhagalov I, Chuck M, Mizushima N, He YW. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. J Exp Med [Internet]. 2007 Jan 22 [cited 2022 Jan 11];204(1):25. Available from: /pmc/articles/PMC2118420/

275. Schmeisser K, Parker JA. Pleiotropic effects of mTOR and autophagy during development and aging. Front Cell Dev Biol. 2019;7(SEP):192.

276. Kimball SR. Integration of signals generated by nutrients, hormones, and exercise in skeletal muscle. Am J Clin Nutr [Internet]. 2014 Jan 1 [cited 2021 Dec 6];99(1):237S. Available from: /pmc/articles/PMC3862457/

277. Liu Z, Jahn LA, Wei L, Long W, Barrett EJ. Amino acids stimulate translation initiation and protein synthesis through an Akt-independent pathway in human skeletal muscle. J Clin Endocrinol Metab [Internet]. 2002 Dec 1 [cited 2022 Mar 8];87(12):5553–8. Available from: https://pubmed.ncbi.nlm.nih.gov/12466352/

278. Neu J, Roig JC, Meetze WH, Veerman M, Carter C, Millsaps M, et al. Enteral glutamine supplementation for very low birth weight infants decreases morbidity. J Pediatr [Internet]. 1997 [cited 2022 May 12];131(5):691–9. Available from: https://pubmed.ncbi.nlm.nih.gov/9403648/

279. Andrews FJ, Griffiths RD. Glutamine: essential for immune nutrition in the critically ill. Br J Nutr [Internet]. 2002 Jan [cited 2022 May 12];87(S1):S3–8. Available from: https://www.cambridge.org/core/journals/british-journal-of-nutrition/article/glutamine-essential-for-immune-nutrition-in-the-critically-ill/541BA13488E23C8AD5EAA5073600C2ED

280. Houdijk APJ, Nijveldt RJ, Van Leeuwen PAM. Glutamine-enriched enteral feeding in trauma patients: Reduced infectious morbidity is not related to changes in endocrine and metabolic responses. J Parenter Enter Nutr. 1999 Sep 1;23(5_suppl):S52–8.

281. Rodriguez PC, Ochoa AC, Al-Khami AA. Arginine metabolism in myeloid cells shapes innate and adaptive immunity. Front Immunol. 2017 Feb 7;8(FEB):93.

282. Milward EA, Shahandeh A, Heidari M, Johnstone DM, Daneshi N, Hondermarck H. Transcriptomics. Encycl Cell Biol. 2016 Jan 1;4:160–5.

283. Tarca AL, Romero R, Draghici S. Analysis of microarray experiments of gene expression profiling. Am J Obstet Gynecol [Internet]. 2006 Aug [cited 2022 Jun 9];195(2):373. Available from: /pmc/articles/PMC2435252/

284. Munder M. Arginase: an emerging key player in the mammalian immune system. Br J Pharmacol [Internet]. 2009 Oct 1 [cited 2022 Jun 9];158(3):638–51. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1476-5381.2009.00291.x

285. Kropf P, Baud D, Marshall SE, Munder M, Mosley A, Fuentes JM, et al. Arginase activity mediates reversible T cell hyporesponsiveness in human pregnancy. Eur J Immunol [Internet]. 2007 [cited 2022 Jun 9];37(4):935–45. Available from: https://pubmed.ncbi.nlm.nih.gov/17330821/

286. Safran M, Rosen N, Twik M, BarShir R, Stein TI, Dahary D, et al. The GeneCards Suite. Pract Guid to Life Sci Databases [Internet]. 2021 [cited 2022 Jun 29];27–56. Available from: https://link.springer.com/chapter/10.1007/978-981-16-5812-9_2

287. Lai Y. A statistical method for the conservative adjustment of false discovery rate (q-value). BMC Bioinformatics [Internet]. 2017 Mar 14 [cited 2022 Aug 30];18(3):155–63. Available from: https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-017-1474-6

288. Yates SC, Zafar A, Hubbard P, Nagy S, Durant S, Bicknell R, et al. Dysfunction of the mTOR pathway is a risk factor for Alzheimer's disease. Acta Neuropathol Commun [Internet]. 2014 Jan 27 [cited 2022 Jul 6];2(1):1–15. Available from: https://actaneurocomms.biomedcentral.com/articles/10.1186/2051-5960-1-3

289. Nathe KE, Parad R, Van Marter LJ, Lund CA, Suter EE, Hernandez-Diaz S, et al. Endotoxin-Directed Innate Immunity in Tracheal Aspirates of Mechanically Ventilated Human Neonates. Pediatr Res 2009 662 [Internet]. 2009 Aug [cited 2022 May 12];66(2):191–6. Available from: https://www.nature.com/articles/pr2009186

290. Nanthakumar N, Meng D, Goldstein AM, Zhu W, Lu L, Uauy R, et al. The mechanism of excessive intestinal inflammation in necrotizing enterocolitis: an immature innate immune response. PLoS One [Internet]. 2011 [cited 2022 May 12];6(3). Available from: https://pubmed.ncbi.nlm.nih.gov/21445298/

291. Saugstad OD, Kwinta P, Wollen EJ, Bik - Multanowski M, Madetko - Talowska A, Jagła M, et al. Impact of antenatal glucocorticosteroids on whole-genome expression in preterm babies. Acta Paediatr [Internet]. 2013 Apr [cited 2022 Jun 15];102(4):349–55. Available from: https://pubmed.ncbi.nlm.nih.gov/23347050/

292. Clish CB. Metabolomics: an emerging but powerful tool for precision medicine. Cold Spring Harb Mol Case Stud [Internet]. 2015 Oct [cited 2022 Jun 9];1(1):a000588. Available from: /pmc/articles/PMC4850886/

293. Krassowski M, Das V, Sahu SK, Misra BB. State of the Field in Multi-Omics Research: From Computational Needs to Data Mining and Sharing. Front Genet. 2020 Dec 10;11:1598.

294. Haas R, Zelezniak A, Iacovacci J, Kamrad S, Townsend SJ, Ralser M. Designing and interpreting 'multi-omic' experiments that may change our understanding of biology. Curr Opin Syst Biol. 2017 Dec 1;6:37–45.

295. Moher D, Shamseer L, Clarke M, Ghersi D, Liberati A, Petticrew M, et al. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. Rev Esp Nutr Humana y Diet [Internet]. 2016 Jan 1 [cited 2022 Jun 21];20(2):148–60. Available from: https://systematicreviewsjournal.biomedcentral.com/articles/10.1186/2046-4053-4-1

296. Heird WC, Hay W, Helms RA, Storm MC, Kashyap S, Dell RB. Pediatric parenteral amino acid mixture in low birth weight infants. Pediatrics. 1988 Jan;81(1):41–50.

Appendix 1 - Amino acid composition of Vaminolact

SUMMARY OF PRODUCT CHARACTERISTICS

1 NAME OF THE MEDICINAL PRODUCT

Vaminolact solution for infusion

2 QUALITATIVE AND QUANTITATIVE COMPOSITION

Amino acids	Amount
Alanine Ph Eur	6.3 grams
Arginine Ph Eur	4.1 grams
Aspartic acid Ph Eur	4.1 grams
Cysteine/Cystine	1.0 grams
Glutamic acid Ph Eur	7.1 grams
Glycine BP	2.1 grams
Histidine USP	2.1 grams
Isoleucine Ph Eur	3.1 grams
Leucine Ph Eur	7.0 grams
Lysine	5.6 grams
Methionine Ph Eur	1.3 grams
Phenylalanine Ph Eur	2.7 grams
Proline Ph Eur	5.6 grams
Serine Ph Eur	3.8 grams
Taurine	0.3 grams
Threonine USP	3.6 grams
Tryptophan USP	1.4 grams
Tyrosine USP	0.5 grams
Valine Ph Eur	3.6 grams

in each 1000 ml
For a full list of excipients, see section 6.1

Product properties

Amino acids	65.3 g/l
Total nitrogen	9.3 g/l corresponding to 58 g/l protein
Acetate	Nil
Energy	240 kcal (1.0 MJ)/l
Osmolality	510 mosmol/kg water
pH	5.2

Free from antioxidant additives, chlorides and other inorganic electrolytes.

Appendix 2 - PRISMA-P checklist (2015) (295)

Section/topic	#	Checklist item	Information reported		Page number(s)
			Yes	No	
ADMINISTRATIVE INFORMATION					
Title					
Identification	1a	Identify the report as a protocol of a systematic review	☒	☐	47
Update	1b	If the protocol is for an update of a previous systematic review, identify as such	☐	☐	n/a
Registration	2	If registered, provide the name of the registry (e.g., PROSPERO) and registration number in the Abstract	☐	☐	n/a
Authors					
Contact	3a	Provide name, institutional affiliation, and e-mail address of all protocol authors; provide physical mailing address of corresponding author	☒	☐	Not applicable for thesis. Refer to abstracts in appendices.
Contributions	3b	Describe contributions of protocol authors and identify the guarantor of the review	☐	☐	See above
Amendments	4	If the protocol represents an amendment of a previously completed or published protocol, identify as such and list changes; otherwise, state plan for documenting important protocol amendments	☐	☐	See above
Support					
Sources	5a	Indicate sources of financial or other support for the review	☒	☐	vi
Sponsor	5b	Provide name for the review funder and/or sponsor	☒	☐	vi

Section/topic	#	Checklist item	Information reported		Page number(s)
			Yes	No	
Role of sponsor/funder	5c	Describe roles of funder(s), sponsor(s), and/or institution(s), if any, in developing the protocol	☐	☒	n/a
INTRODUCTION					
Rationale	6	Describe the rationale for the review in the context of what is already known	☒	☐	46
Objectives	7	Provide an explicit statement of the question(s) the review will address with reference to participants, interventions, comparators, and outcomes (PICO)	☒	☐	47 - 49
METHODS					
Eligibility criteria	8	Specify the study characteristics (e.g., PICO, study design, setting, time frame) and report characteristics (e.g., years considered, language, publication status) to be used as criteria for eligibility for the review	☒	☐	53 - 54
Information sources	9	Describe all intended information sources (e.g., electronic databases, contact with study authors, trial registers, or other grey literature sources) with planned dates of coverage	☒	☐	47
Search strategy	10	Present draft of search strategy to be used for at least one electronic database, including planned limits, such that it could be repeated	☒	☐	49 - 52
STUDY RECORDS					
Data management	11a	Describe the mechanism(s) that will be used to manage records and data throughout the review	☒	☐	55
Selection process	11b	State the process that will be used for selecting studies (e.g., two independent reviewers) through each phase of the review (i.e., screening, eligibility, and inclusion in meta-analysis)	☒	☐	58 - 59

Section/topic	#	Checklist item	Information reported		Page number(s)
			Yes	No	
Data collection process	11c	Describe planned method of extracting data from reports (e.g., piloting forms, done independently, in duplicate), any processes for obtaining and confirming data from investigators	☒	☐	55 - 56
Data items	12	List and define all variables for which data will be sought (e.g., PICO items, funding sources), any pre-planned data assumptions and simplifications	☒	☐	55, 56, Appendix 4
Outcomes and prioritization	13	List and define all outcomes for which data will be sought, including prioritization of main and additional outcomes, with rationale	☒	☐	46
Risk of bias in individual studies	14	Describe anticipated methods for assessing risk of bias of individual studies, including whether this will be done at the outcome or study level, or both; state how this information will be used in data synthesis	☒	☐	66 - 67
DATA					
Synthesis	15a	Describe criteria under which study data will be quantitatively synthesized	☒	☐	57
	15b	If data are appropriate for quantitative synthesis, describe planned summary measures, methods of handling data, and methods of combining data from studies, including any planned exploration of consistency (e.g., I^2, Kendall's tau)	☒	☐	57
	15c	Describe any proposed additional analyses (e.g., sensitivity or subgroup analyses, meta-regression)	☒	☐	46, 63-65
	15d	If quantitative synthesis is not appropriate, describe the type of summary planned	☐	☐	n/a
Meta-bias(es)	16	Specify any planned assessment of meta-bias(es) (e.g., publication bias across studies, selective reporting within studies)	☐	☐	n/a
Confidence in cumulative evidence	17	Describe how the strength of the body of evidence will be assessed (e.g., GRADE)	☒	☐	66

Appendix 3 - Completed data extraction sheet

Article	Title (First author)	Location	Study Design	Sample size	Groups	Gestational Age	AA solution	AA intake at sampling (g/kg/d)	Primary outcome	Secondary outcome(s)	Plasma Leucine (µmol/L)	Reference Range	Method of analysis	Sampling time
1	Early Changes in Plasma Amino Acid Concentrations during Aggressive Nutritional Therapy in Extremely Low Birth Weight Infants C. Blanco et al. (2011)	University Hospital, San Antonio, Texas	Randomised, double-masked prospective trial	61	Early and high AA protocol (n = 30) 2 g/kg/d of intravenous AA soon after birth and advanced by 1 g/kg every day to 4 g/kg/d	25.7 ± 2.0	Aminosyn PF (Abbott Laboratories)	3.7 ± 0.9	Changes in plasma AA levels	BUN, Ammonia	[a] 202.6 (136.8 - 277.5)	-	Reverse phase, HPLC using Waters PICO-Tag method	Days 1,3 and 7 of PN
					Standard AA protocol (n = 31) 0.5 g/kg/d started between 23-36 hours after birth and increased by 0.5 g/kg every day to a maximum of 3 g/kg/d	26.3 ± 2.0		2.9 ± 0.2			[a] 192.5 (156.0 - 235.3)			

Article	Title (First author)	Location	Study Design	Sample size	Groups	Gestational Age	AA solution	AA intake at sampling (g/kg/d)	Primary outcome	Secondary outcome(s)	Plasma Leucine (µmol/L)	Reference Range	Method of analysis	Sampling time
2	Effect of low versus high early parenteral nutrition on plasma amino acid profiles in very low birthweight infants A. Bulbul et al. (2012)	Sisli Children Hospital, Istanbul, Turkey	Prospective, randomised clinical cohort study	44	Group 1: Early, low dose PN (n = 22) 1.0 g/kg/d of AA and lipid, increasing by 1.0 g/ kg/day, with an aimed intake of 3.0 g/kg/d amino acids and 3 g/ kg/d lipid on the third day of postnatal life	29.4 ± 1.8	Primene 10% (Baxter/ Clintech)	3.6	Plasma AA concentrations in the first two weeks	Blood ammonia, BUN, triglyceride concentrations, postnatal growth	100.0 ± 28.4	Term, breast-fed infants (111.3 ± 27.3) (226) Preterm, human-milk-fed infants (87 ± 14) (228) LBWI receiving TrophAmine (101.5 ± 36.4) (296) Cord blood of neonates at 29 weeks gestational age (153 ± 36) (229) ELBW infants (63 ± 30.4) (126)	HPLC with an automated amino acid analyser (Shimadzu CLASS-VP V 6.12 SP1)	Baseline (before PN), 7th and 14th postnatal day
					Group 2: Early, high dose PN (n = 22) 3.0 g/kg/d of AA and lipid, starting on the first day of postnatal life	29.1 ± 1.1		3.6			109.0 ± 72.4			

Article	Title (First author)	Location	Study Design	Sample size	Groups	Gestational Age	AA solution	AA intake at sampling (g/kg/d)	Primary outcome	Secondary outcome(s)	Plasma Leucine (µmol/L)	Reference Range	Method of analysis	Sampling time
3	Effect of amino acid composition of parenteral solutions on nitrogen retention and metabolic response in very-low-birth weight infants P. Chessex et al. (1985)	Perinatal Service and Research Center, Ste. Justine Hospital, and the Department of Pediatrics, University of Montreal	Crossover study, randomised design	15	Single group - Each infant was studied twice over two consecutive 6 day study periods receiving the two different PN solutions Group 1: Started with Travasol (n = 7) Group 2: Started with Vamin (n = 8)	28 ± 1	Travasol 10% blend B (Travasol-Baxter Laboratories) Vamin 7% (Pharmacia)	2.7	N balance	Plasma AA concentrations, growth rate	Group 1: 71 ± 18 Group 2: 106 ± 23	-	IEC using a modified Beckman 121 amino acid analyser	Day 6 of each regimen, between 9am and 11am
4	Glutamine supplement with parenteral nutrition decreases whole body proteolysis in low birth weight infants S. Kalhan et al. (2005)	Schwartz Center for Metabolism and Nutrition, MetroHealth Medical Center, Department of Pediatrics and Biochemistry, Case Western Reserve University, Cleveland, Ohio	Randomised Control Trial	20	Glutamine group (n = 10) Control group (n = 10)	26.7 ± 1.6 27.7 ± 2.0	TrophAmine 10% (McGaw)	3.2 ± 1.1 2.5 ± 0.8	To examine the effect of supplemental glutamine on whole body protein/ nitrogen and glutamine kinetics	Plasma AA concentrations, acid base status, blood ammonia, BUN	109.1 ± 21.4 124.6 ± 22.17	-	HPLC	After 3 to 5 days of PN, tracer studies were performed. Blood samples were obtained at isotopic steady state between 165 and 300 mins of tracer infusion.

Article	Title (First author)	Location	Study Design	Sample size	Groups	Gestational Age	AA solution	AA intake at sampling (g/kg/d)	Primary outcome	Secondary outcome(s)	Plasma Leucine (µmol/L)	Reference Range	Method of analysis	Sampling time
5	Effect of Hyperalimentation and Insulin-Treated Hyperglycemia on Tyrosine Levels in Very Preterm Infants Receiving Parenteral Nutrition K. Mayes et al. (2014)	Liverpool Women's Hospital, Liverpool, UK	Randomised Control Trial	142 (118 AA profiles)	Hyperalimentation group (n = 68) 20% more energy (117 kcal/kg/d), proportional increase in dextrose (16.3 g/kg/d), protein (4 g/kg/d) and fat (4 g/kg/d) PN increased over 7 days	26 ± 1.5	Primene 10% (Baxter/ Clintech)	PN protein the 2d before AA sampling = 5.6 ± 1.7	To compare plasma AA profiles on days 8-10 of life in preterm infants receiving intervention versus control regimen	–	130 (104-156)	97 (44–169) Obtained from a multicentre U.K. study of infants <6 months using the same analysis technique (174)	IEC	Days 8-10 of life
					Control group (n = 74) 3 kcal/kg/d with dextrose (13.5 g/kg/d), protein (3 g/kg/d), and fat (3 g/kg/d) PN increased over 5 days	26.2 ± 1.5		PN protein the 2d before AA sampling = 4.4 ± 1.7			111 (86-129)			

Article	Title (First author)	Location	Study Design	Sample size	Groups	Gestational Age	AA solution	AA intake at sampling (g/kg/d)	Primary outcome	Secondary outcome(s)	Plasma Leucine (µmol/L)	Reference Range	Method of analysis	Sampling time
6	Plasma amino acid profiles in preterm infants receiving Vamin 9 glucose or Vamin Infant S. Mitton et al. (1992)	Charing Cross and Westminster Medical School, London, UK	Open prospective study. Randomly assigned to PN solution.	29	Vamin 9 glucose (n =18)	29 ± 3	Vamin 9 glucose (Kabi Pharmacia, UK)	3.2	Unclear primary Plasma AA levels and N retention	-	[b] 125 (51 - 204)	Breast-fed infants (226) 111 (53 – 169)	IEC using a LKB α-amino acid analyser and a lithium buffer system	Between 8-10am on day 5 of PN
					Vamin Infant (n = 11)	29 ± 2	Vamin Infant (Kabi Pharmacia, UK)	3.2			[b] 135 (88 - 195)			
7	High Protein Intake Does Not Prevent Low Plasma Levels of Conditionally Essential Amino Acids in Very Preterm Infants Receiving Parenteral Nutrition C. Morgan and L. Burgess (2015)	Liverpool Women's Hospital, Liverpool, UK	Single-centre, Randomised Control Study	150 (126 AA profiles)	SCAMP (n = 74) Designed to provide 3.8 g/kg/d of protein (4.3 g/kg/d of AA) and 105 kcal/kg/day)	26.8 ± 1.3	Vaminolact (Fresenius-Kabi)	In the 48 hours prior to sample, total protein intake was 3.66 ± 0.63) g/kg/d	To compare plasma AA profiles at approximately day 9 in SCAMP versus control	-	156 (131 - 169)	97 (44–169) Obtained from a multicentre U.K. study of infants <6 months using the same analysis technique (174)	IEC	Median (IQR) age of 9 (8-10) days
					Control group (n = 76) Standardized, concentrated neonatal PN regimen used in current clinical practice. Designed to provide 2.8g/kg/d of protein (3.3 g/kg/d of AA) and 85 kcal/kg/day energy	26.6 ± 1.4		In the 48 hours prior to sample, total protein intake was 3.03 ± 0.33) g/kg/d			136 (122 - 156)			

Article	Title (First author)	Location	Study Design	Sample size	Groups	Gestational Age	AA solution	AA intake at sampling (g/kg/d)	Primary outcome	Secondary outcome(s)	Plasma Leucine (µmol/L)	Reference Range	Method of analysis	Sampling time
8	Clinical trial of a 6.5% amino acid infusion in appropriate-for-gestational-age premature neonates E. Ogata et al. (1983)	Northwestern Memorial Hospital, Chicago, USA	Randomised Trial	17	New solution (Neopham) with increased cysteine, tyrosine and histidine and reduced NEAA (n = 9)	28.0 ± 1.6	Neopham (Cutter Laboratories)	2.56 ± 0.39	Plasma AA levels	Body weight, length and skinfold thickness. Biochemical and hematologic monitoring	111 ± 39	-	Unknown, used the method of Heird and Winters 1986	Before AA infusion, and after 1 and 2 weeks of infusion
					Standard solution (Aminosyn) (n = 8)	28.2 ± 0.7	Aminosyn (Abbott Laboratories)	2.69 ± 0.47			91 ± 20			
9	Total Parenteral Nutrition in Very Low Birth Weight Infants with Travasol 10% Blend C M Pineault et al. (1986)	St. Justine's Hospital, Montreal, Quebec, Canada	Cohort study	10	Single group	27 ± 0.5	Travasol 10% blend C (Travenol-Baxter Laboratories)	2.61 ± 0.02	Plasma AA levels	Blood gas, electrolyte, glucose and BUN	[c] 68 ± 4	-	IEC with a modified Beckman 121 amino acid analyser	Between 9-11am After 4.6 ± 0.3 days of AA infusion

Article	Title (First author)	Location	Study Design	Sample size	Groups	Gestational Age	AA solution	AA intake at sampling (g/kg/d)	Primary outcome	Secondary outcome(s)	Plasma Leucine (µmol/L)	Reference Range	Method of analysis	Sampling time
10	Effect of parenteral glutamine supplementation on plasma amino acid concentrations in extremely low-birth-weight infants B. Poindexter et al. (2003)	14 participating National Institute of Child Health and Human Development Neonatal Research Network centres (USA)	Multicentre Randomised Control Trial	141	Glutamine group (n = 72) Isonitrogenous study amino acid solution with 20% glutamine; this solution consisted of TrophAmine and a nonpyrogenic L-glutamine powder	26.2 ± 2.0	TrophAmine (B. Braun)	2.40 ± 0.91	Effect of parenteral glutamine supplement on plasma AA concentrations	Plasma ammonia	100 (80 - 126)	Term, breast-fed infants (111.3 ± 27.3) (226) Preterm, human-milk-fed infants (87 ± 14) (228) Neonates at 29 weeks gestational age (153 ± 36) (229) LBWI receiving TrophAmine (101.5 ± 36.4) (296)	IEC with post-column Ninhydrin detection and an automated amino acid analyser (model 6300; Beckman Instrument, Fullerton, CA)	Before initiation of study PN and after the infant had received study PN for about 10 days
					Control group (n = 69) TrophAmine	26.3 ± 1.8		2.19 ± 1.09			118 (89 - 142)			

Article	Title (First author)	Location	Study Design	Sample size	Groups	Gestational Age	AA solution	AA intake at sampling (g/kg/d)	Primary outcome	Secondary outcome(s)	Plasma Leucine (µmol/L)	Reference Range	Method of analysis	Sampling time
11	Evaluation of a taurine containing amino acid solution in parenteral nutrition L. Thornton and E Griffin (1991)	Coombe Lying-In Hospital, Dublin	Non-randomised trial (the 15 infants receiving Vaminolact were compared to the 10 previous infants who received Vamin glucose)	25	Single group relevant to this review (Vamin glucose infants excluded because the mean gestation >32 weeks) (n = 15)	29.7 ± 3.6	Vaminolact (KabiVitrum, sold as Vamin Infant in the UK)	2.3 ± 0.2	Plasma AA levels	Other biochemical data, nitrogen balance, body weight	166 ± 66	-	Ion-exchange chromatography with a Locarte amino acid analyser	Before starting study PN, after 24 hours of an intake of 2g/kg/d of AA, and again after three days 1on an intake of 2-5 g/kg/d of AA
12	Amino acid solutions for premature neonates during the first week of life: the role of N-acetyl-L-cysteine and N-acetyl-L-tyrosine J. Van Goudoever et al. (1994)	University Hospital Rotterdam and Sophia Children's Hospital Rotterdam	Randomised trial	20	Aminovenos (n = 10)	31 ± 2	Aminovenös-N-päd 10% (Fresenius AG)	2.3 ± 0.2	Compare different AA solutions by measuring plasma AA. Special attention on tyrosine and cysteine	Nitrogen retention and urinary AA	86 ± 19	Breast-fed, term neonates (226) 53 – 169	IEC with LKB 4151 Alpha+ Amino Acid Analyzer (Pharmacia LKB Biochrom Ltd, Cambridge, UK) and LC 5000 Biotronic (Munich Germany)	Postnatal day 7
					Vaminolact (n = 4)	30 ± 2	Vaminolact 6.5% (Kabi Pharmacia)	2.1 ± 0.2			102 ± 23			
					Primene (n = 6)	30 ± 2	Primene 10% (Clintec)	2.2 ± 0.3			119 ± 29			

Results are expressed as mean ± standard deviation (SD) or median (Q_1 –Q_3) unless indicated otherwise:

[a] Median (10^{th} to 90^{th} percentile range)

[b] Mean (95% confidence intervals)

[c] Mean ± SEM

Appendix 4 - Quality Assessment Tool

Title:

Author:

Question	Descriptor	n/a	n/r	Yes	Further details
1. Internal Validity					
1.1. Does the title summarise the study?					
1.2. Does the abstract provide a clear summary of the study?					
1.3. Introduction/background: - Population clearly defined - Incidence given of condition that caused PN dependency - Driver for this study identified	VPN - Gestational age and postnatal age provided				
	Describes the rationale behind the prescription of PN in study participants				
	Explanation as to why this particular study is being undertaken				
1.4. Was the primary aim/objective clearly described?					

Question	Descriptor	n/a	n/r	Yes	Further details
1.5. Were any secondary aims/objectives clearly described?					
1.6. Was the hypothesis clearly stated?					
1.7. Was a sample size determination calculation completed?					
1.8. Were the eligibility criteria for study participants clear?					
1.9. Were the rejection criteria for study participants clear?					
1.10. Clear rationale for choice of PN/AA solution given					
1.11. Was ethical approval obtained and described clearly?					
1.12. Was patient consent obtained and reported clearly?					

Question	Descriptor	n/a	n/r	Yes	Further details
1.13. Were any co-existing conditions in patients reported?					
1.14. Were any baseline data obtained and measurements reported?					
1.15. Were adverse events considered?					
1.16. Were adverse events clearly reported?					
1.17. Was there statement on funding statement? If so, were any funding sources disclosed?					
1.18. Was there a conflict of interest statement? If so, were any potential conflicts of interest identified and adequately explained?					
2. Randomisation					
2.1. Was the sequence generation explained?					

Question	Descriptor	n/a	n/r	Yes	Further details
2.2. Was the allocation concealment described and adequate?					
2.3. Was the blinding of participants, clinical staff and outcome assessors appropriate?					
3. Crossover Studies					
3.1. Was the order of the treatments randomised?					
3.2. Was it clear how many treatments or study periods were used?					
3.3. Was a suitable wash-out period used?	Needed to avoid bias from a possible carry-over effect when changing from a period of one AA solution to the next.				
3.4. Were drop-outs reported and considered acceptable?	Possible risk of bias where participants received one treatment but not the second				
3.5. Was a paired analysis completed?	Needed to account for within person differences				

Question	Descriptor	n/a	n/r	Yes	Further details
4. Manipulation					
4.1. AA solution: - Brand name - Manufacturer/company - Strength - Composition provided					
4.2. Was the route of PN administration reported?	Peripheral versus central				
4.3. Was the duration of PN described/ reported?					
4.4. Was the composition of the non-protein components of the PN solution described?	Description of the source and intake potentially including the non-protein caloric intake.				
4.5. Were any other sources of nutrients reported? (Formula/oral feeds)					
4.6. Was the timing of plasma AA analysis clearly stated?					
5. MEASUREMENT METHODOLOGY					
5.1. Was the AA analysis method clearly reported? (IEC versus HPLC)					

Question	Descriptor	n/a	n/r	Yes	Further details
5.2. Was the type of blood sample described?	Plasma sample versus DBS				
5.3. Were the instruments used to measure plasma AA described?	Sufficient explanation of the method to allow for replication.				
5.4. Was the data completely reported?	No if high drop-out rates, outcomes reported only for some groups of participants, pre-specified outcomes not reported.				
5.5. Were any confounding factors identified?					
5.6. Were the confounding factors considered in the study design/analysis?					
5.7. Was the patient follow-up sufficient?	The effects should have been monitored long enough to reveal themselves.				
6. External Validity					
6.1. Are the results and conclusions relevant to the aims/objectives for the study?					

Question	Descriptor	n/a	n/r	Yes	Further details
6.2. Are the results critically appraised in relation to previous work?	Previous studies that support or report similar findings				
6.3. Are the results applicable to the population of the systematic review?	Are the findings applicable to other patients in the population of study (VPN)?				
6.4. Does the study demonstrate: - Proof of concept? - Efficacy?	Taking account of design and achieved sample size				

Appendix 5 - Quality assessment tool findings from articles in the systematic review

QUESTIONS		ARTICLE NUMBER												PERCENTAGE SCORE PER QUESTION
		1	2	3	4	5	6	7	8	9	10	11	12	
1. INTERNAL VALIDITY	1.1. Does the title summarise the study?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
	1.2. Does the abstract provide a clear summary of the study?	Y	Y	Y	Y	Y	Y	Y	n/a	Y	Y	Y	Y	100%
	1.3. Introduction/background:	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
	- Population clearly defined													
	- Incidence given of condition that caused PN dependency	N	N	N	N	N	N	N	N	N	N	N	N	0%
	- Driver for this study identified	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
	1.4. Was the primary aim/objective clearly described?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
	1.5. Were any secondary aims/objectives clearly described?	n/a	Y	n/a	n/a	n/a	n/a	n/a	n/a	n/a	Y	n/a	n/a	100%
	1.6. Was the hypothesis clearly stated?	N	Y	N	N	Y	N	Y	N	N	Y	Y	N	41.7%
	1.7. Was a sample size determination calculation completed?	Y	Y	N	N	Y	N	Y	N	N	N	N	N	33.3%
	1.8. Were the eligibility criteria for study participants clear?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
	1.9. Were the rejection criteria for study participants clear?	Y	Y	N	N	Y	Y	Y	Y	N	Y	N	N	58.3%
	1.10. Clear rationale for choice of PN/AA solution given	Y	N	Y	N	N	Y	N	Y	Y	N	Y	Y	58.3%
	1.11. Was ethical approval obtained and described clearly?	Y	Y	N	Y	Y	Y	Y	N	Y	Y	N	Y	75%
	1.12. Was patient consent obtained and reported clearly?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%

	1.13. Were any co-existing conditions in patients reported?	N	Y	Y	N	N	Y	N	Y	Y	N	Y	N	50%
	1.14. Were any baseline data obtained and measurements reported?	N	Y	N	N	N	N	N	Y	N	Y	Y	N	33.3%
	1.15. Were adverse events considered?	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	N	83.3%
	1.16. Were adverse events clearly reported?	Y	Y	Y	Y	Y	N	Y	Y	N	Y	Y	N	75%
	1.17. Was there statement on funding statement?	N	Y	Y	Y	Y	N	Y	N	Y	Y	N	N	58.3%
	If so, were any funding sources disclosed?	n/a	N	Y	Y	N	n/a	Y	n/a	Y	N	N	n/a	50%
	1.18. Was there a conflict of interest statement?	Y	Y	Y	N	N	N	N	N	N	Y	Y	N	41.6%
	If so, were any potential conflicts of interest identified and adequately explained?	n/a	n/a	N	n/a	n/a	n/a	n/a	n/a	n/a	N	Y	n/a	33.3%
2. RANDOMISATION	2.1. Was the sequence generation explained?	N	Y	n/a	N	Y	N	Y	N	n/a	Y	n/a	N	44.4%
	2.2. Was the allocation concealment described?	Y	Y	n/a	N	Y	N	Y	Y	n/a	Y	n/a	N	66.6%
	2.3. Was the blinding of participants, clinical staff and outcome assessors appropriate?	Y	Y	n/a	N	Y	N	Y	Y	n/a	Y	n/a	N	66.6%
3. CROSSOVER STUDIES	3.1. Was the order of the treatments randomised?	n/a	n/a	Y	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	100%
	3.2. Was it clear how many treatments or study periods were used?	n/a	n/a	Y	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	100%
	3.3. Was a suitable wash-out period used?	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	-
	3.4. Were drop-outs reported and considered acceptable?	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	-
	3.5. Was a paired analysis completed?	n/a	n/a	Y	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	100%

4. MANIPULATION	4.1. AA solution: - Brand name - Manufacturer/company - Strength - Composition provided	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
		Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
		n/a	Y	Y	Y	Y	n/a	Y	n/a	Y	n/a	n/a	Y	100%
		N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	58.3%
	4.2. Was the route of PN administration reported?	N	Y	Y	N	N	N	N	Y	Y	N	Y	Y	50%
	4.3. Was the duration of PN described/ reported?	Y	Y	Y	N	Y	N	Y	Y	N	N	Y	N	58.3%
	4.4. Was the composition of the non-protein components of the PN solution described?	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	91.7%
	4.5. Were any other sources of nutrients reported? (Formula/oral feeds)	N	Y	N	N	Y	n/a	Y	n/a	N	Y	N	n/a	44.4%
	4.6. Was the timing of plasma AA analysis clearly stated?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
5. MEASUREMENT METHODOLOGY	5.1. Was the AA analysis method clearly reported? (IEC versus HPLC)	Y	Y	N	N	Y	Y	Y	N	N	Y	Y	N	58.3%
	5.2. Was the type of blood sample described?	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	91.7%
	5.3. Were the instruments used to measure plasma AA described?	N	Y	Y	N	N	Y	N	N	Y	Y	Y	Y	58.3%
	5.4. Was the data completely reported?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
	5.5. Were any confounding factors identified?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
	5.6. Were the confounding factors considered in the study design/analysis?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
	5.7. Was the patient follow-up sufficient?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%

6. EXTERNAL VALIDITY	6.1. Are the results and conclusions relevant to the aims/objectives for the study?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
	6.2. Are the results critically appraised in relation to previous work?	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	91.7%
	6.3. Are the results applicable to the population of the systematic review?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
	6.4. Does the study demonstrate: - Proof of concept?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
	- Efficacy?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
Numerical Score		32	41	35	27	34	29	35	30	30	36	35	27	-
Total Score		42	45	45	44	44	41	44	40	41	45	41	42	-
Percentage Score Per Paper		76.2	91.1	77.8	61.4	77.3	70.7	79.5	75.0	73.2	80.0	85.4	64.3	76.0

Appendix 6 - RCPCH Conference Abstract

Title: The relationship between parenteral leucine intake and plasma leucine in very preterm infants dependent on parenteral nutrition. A systematic review.

Authors: K. Davies [1], C. Morgan [2]

Affiliations:

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[2] Neonatal Intensive Care Unit, Liverpool Women's Hospital, Liverpool, UK

Aims:

Very preterm neonates (VPNs) are dependent on parenteral nutrition (PN) from birth. Recent evidence from sick term infants indicates that immediate provision of parenteral amino acids (AA) may be harmful and increase the risk of infection (1). The inhibition of autophagy, a catabolic process important in the developing immune system has been proposed as a mechanism. Current parenteral AA formulations used routinely in VPN result in high plasma essential AA (including leucine) levels, well above the normal reference range (2). Leucine is known to be a potent activator of the mTOR pathway, which increases protein synthesis but inhibits autophagy. We have previously shown how a systematic review of published plasma AA data can be used to calculate the optimal (higher) neonatal PN content of a single deficient AA (3). We adapted this methodology to investigate the relationship between PN leucine intake and plasma leucine levels in VPN. The aim was to quantify the relationship between parenteral leucine intake and plasma leucine concentration to allow the optimal (lower) leucine content of neonatal PN to be calculated. This would also provide a methodology to correct the overprovision of the other essential AA in neonatal PN.

Methods:

The four databases searched were Cochrane, PubMed, Scopus and Web of Science. The systematic review was prepared following PRISMA-P 2015 guidelines. The PICO format was used to develop the search strategy. The population of interest to this review was PN-dependent VPNs; dependency was defined as requiring a minimum of 7 days of PN. The intervention and comparator are low versus high parenteral leucine intakes expressed as either the percentage leucine content of the AA solution (%) or absolute leucine intake (mg/kg/d). The outcome measured was plasma leucine concentration (µmol/L). All study designs were eligible for inclusion, except for review articles. Only articles which reported the actual parenteral AA intake and measured plasma leucine concentration after day 3 (72 hours) of PN were eligible. The data were obtained using a data extraction form designed for this review. Quality assessments using a custom-designed tool and the GRADE framework were performed.

Results:

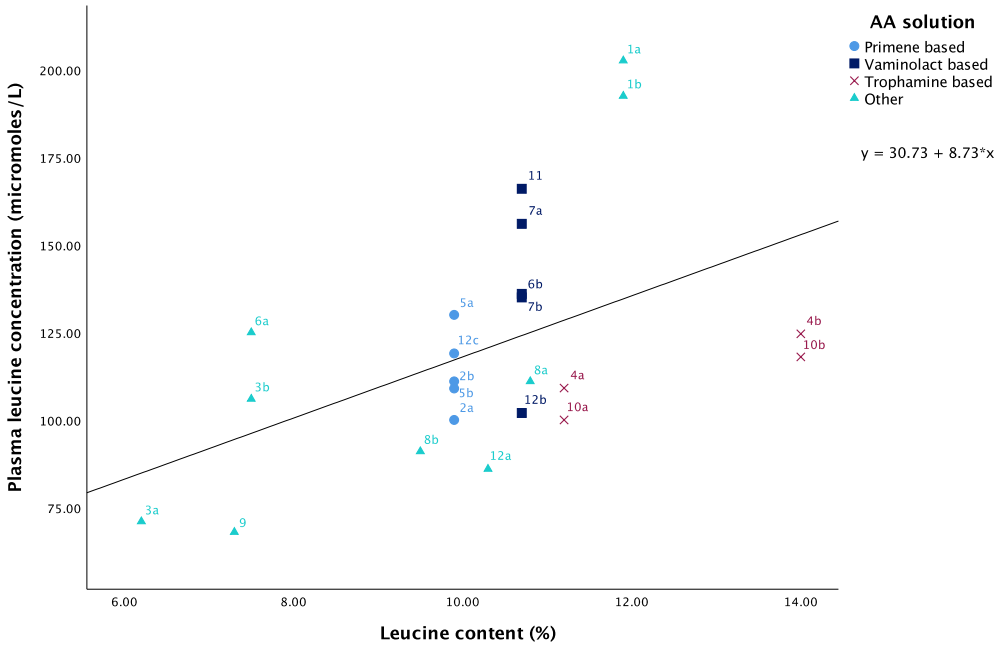
Twelve articles met the inclusion criteria, which collectively studied 650 VPNs. The dose-concentration relationships of leucine content (%) and absolute leucine intake (g/kg/d) with plasma leucine concentration (µmol/L) both showed significant, moderately positive correlations ($p < 0.05$), as shown in Figure 1. Regression analysis indicated that absolute leucine intake was the stronger predictor of outcome. Subgroup analysis indicated that neither the overall study design nor the analytical method used for AA analysis affected the plasma leucine level.

Conclusions:

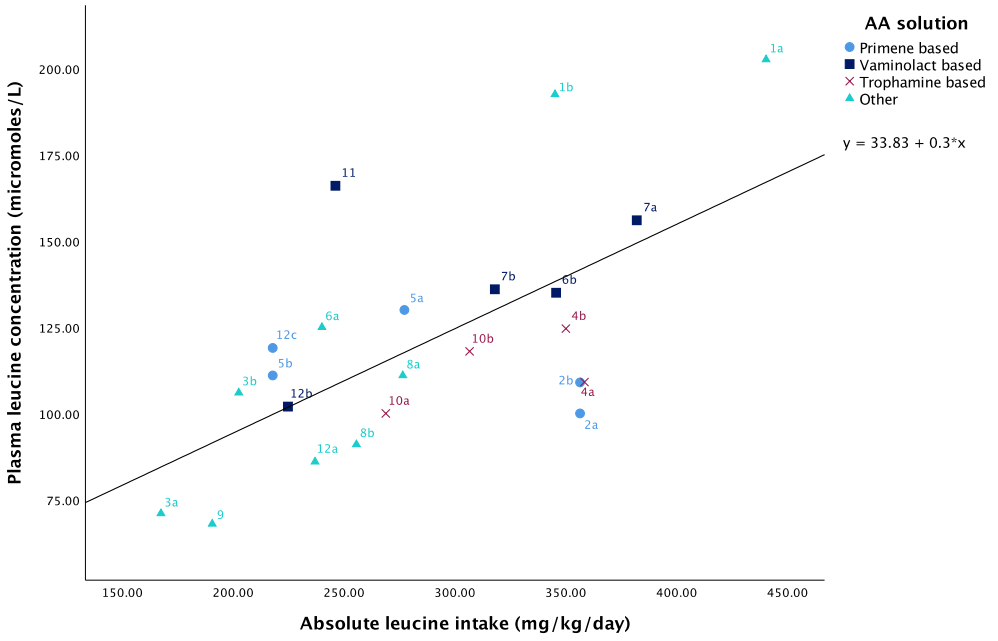
There is a linear relationship between plasma leucine concentration and both percentage leucine content and leucine intake (mg/kg/d) in VPNs. This work indicates that the leucine content of future neonatal PN solutions should be reduced to 8-9g/100gAA to achieve plasma AA concentrations within the reference range. This methodology can be applied to all essential AA.

Figure 1:

Dose concentration relationship graph of percentage leucine content with plasma leucine concentration



Dose concentration relationship graph of percentage leucine content with plasma leucine concentration



References:

(1) Lancet Child & Adolescent Health, van Puffelen E et al.

(2) JPEN, Morgan, C. & Burgess, L

(3) Nutrition Reviews, Premakumar CM et al.

Appendix 7 - RCPCH Conference e-Poster

The relationship between parenteral leucine intake and plasma leucine in very preterm infants dependent on parenteral nutrition. A systematic review.

K. Davies, Department of Women's and Children's Health, University of Liverpool
C. Morgan, Department of Neonatology, Liverpool Women's Hospital

AIMS

Very preterm neonates (VPN) are dependent on parenteral nutrition (PN) from birth. Current parenteral amino acid (AA) formulations result in plasma essential AA levels (including leucine) above the reference range. Leucine is a potent activator of the mTOR pathway, increasing protein synthesis but inhibiting autophagy, a catabolic process important in innate immunity.

1. Quantify the relationship between parenteral leucine intake and plasma leucine level to allow calculation of the optimal (lower) leucine content of neonatal PN

2. Investigate the effect of study design and different AA chromatography techniques

METHODS

Databases:
Cochrane, PubMed, Scopus and Web of Science

Inclusion Criteria:
- Human subjects
- Babies born ≤32 weeks (VPN), studied for the first 28 days of life (neonatal period)
- Infants received a minimum of 7 days PN
- Named AA solution with known % leucine content
- Actual (vs prescribed) protein/AA intake reported
- Plasma leucine reported after day 3 (72h) of PN
- All study designs, except for review articles.

Quality assessment was performed using a custom quality assessment tool and the GRADE framework.

RESULTS

12 articles met the inclusion criteria (n = 650 VPN)
- Nine RCTs, one non-randomised control trial, one cohort study and a cross-over study
- Studies were published between 1983 and 2017
- Sample sizes ranged from 15 to 141 VPNs
- Infants had a minimum and maximum gestational age of 23.7 and 33 weeks

The dose-concentration relationships of % leucine content and absolute leucine intake (g/kg/d) with plasma leucine concentration (µmoles/L) showed significant, moderate, positive correlations ($p < 0.01$).

Regression analysis indicated absolute intake was a stronger predictor ($p < 0.05$).

Figure 1 - Dose concentration relationship graph of absolute leucine intake with plasma leucine concentration

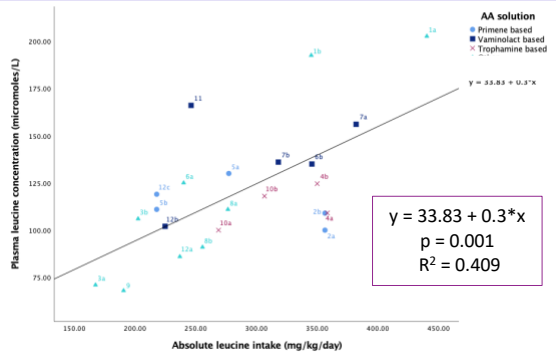
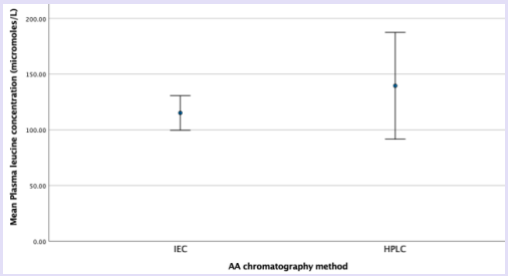


Figure 2 – Simple error bar of mean plasma leucine concentration by AA chromatography method



8 articles used ion-exchange chromatography (IEC) and 3 used high-performance liquid chromatography (HPLC)

The difference in plasma leucine between analysis methods was not significant (Figure 2).

CONCLUSION

A linear relationship exists between plasma leucine concentration and both percentage leucine content and leucine intake in VPN.

Using the dose-concentration relationship, the leucine content of neonatal PN should be reduced to 8-9g/100g AA to achieve reference plasma AA levels.

Our methodology offers a systematic approach to rebalancing PN formulations using the existing evidence base which could be replicated for the remaining AA.

Morgan, C., & Burgess, L. (2017). High Protein Intake Does Not Prevent Low Plasma Levels of Conditionally Essential Amino Acids in Very Preterm Infants Receiving Parenteral Nutrition. JPEN
Premakumar CM et al. Relationship between arginine intake in parenteral nutrition and preterm neonatal population plasma arginine concentrations: a systematic review. Nutr Rev

Appendix 8 - EAPS Conference Abstract

Title: The relationship between parenteral leucine intake and plasma leucine in very preterm infants dependent on parenteral nutrition. A systematic review.

Authors: K. Davies [1], C. Morgan [2]

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Keywords: Leucine, Parenteral Nutrition, Very Preterm Neonates

Background and aims:

Very preterm neonates (VPN) are dependent on parenteral nutrition (PN) from birth. Recent evidence from sick term infants indicates immediate provision of parenteral amino acids (AA) may increase infection risk. Inhibition of autophagy is a proposed mechanism. Leucine is a potent activator of the mTOR pathway, increasing protein synthesis but inhibiting autophagy. High plasma essential AA levels (including leucine) suggest overprovision by parenteral AA formulations. We have published systemic review methodology that calculates the optimal content of arginine, a deficient AA. This was adapted to investigate the relationship between parenteral leucine intake and plasma leucine levels in VPN.

Methods:

Cochrane, PubMed, Scopus and Web of Science were searched regardless of study design, excluding review articles. Articles reporting actual parenteral AA intake and plasma leucine concentration after day 3 were eligible. A data extraction form was designed for this review. Quality assessment used a custom-designed tool and the GRADE framework.

Results:

Twelve articles met inclusion criteria (n=650 VPN). The dose-concentration relationships of leucine content (%) and absolute leucine intake (g/kg/d) with plasma leucine concentration (µmoles/L) showed significant, moderate, positive correlations ($p<0.05$). Regression analysis indicated absolute intake was a stronger predictor. Subgroup analysis revealed neither study design nor the analytical method used for AA analysis affected plasma leucine levels.

Conclusions:

In VPN, there is a linear relationship between plasma leucine level and both leucine content (%) and absolute intake. Leucine content of neonatal PN should be reduced to 8-9g/100g AA to achieve reference plasma AA levels. The methodology can be applied to all essential AA.

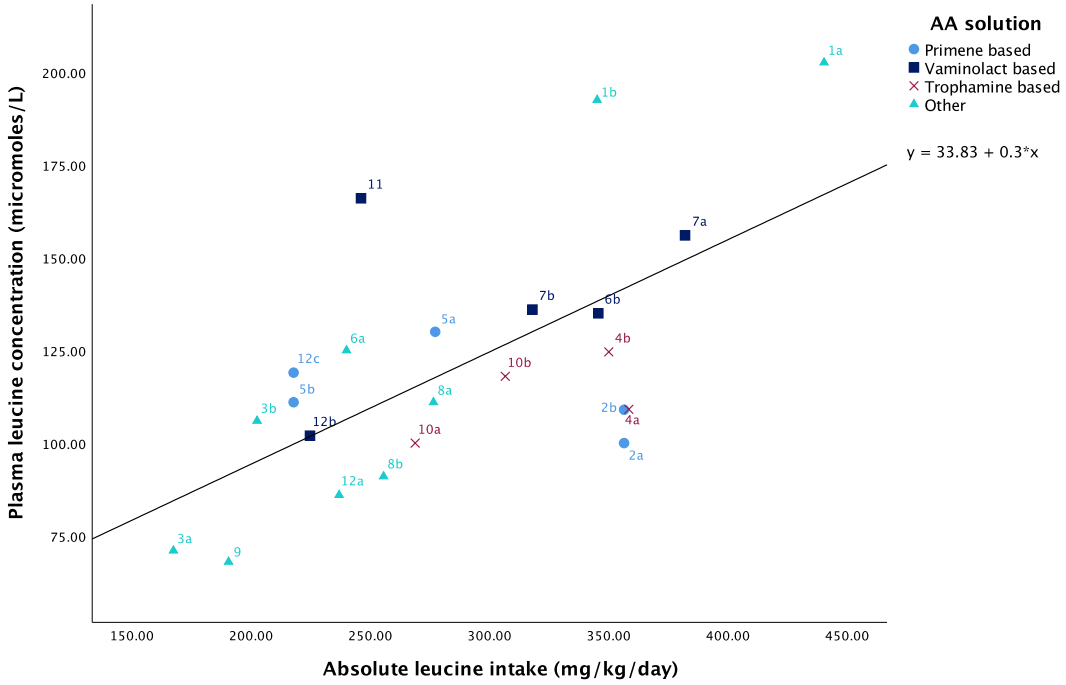

Figure 1 - Dose concentration relationship graph of absolute leucine intake with plasma leucine concentration

An exploratory study of increased **P**reterm **A**rginine **INT**ake on biological pathways affecting immune function in infants requiring early parenteral nutrition

Protocol

IRAS ID: 253730

SPONSOR ID: LWH

Exploratory physiological study

Version 1.0 (amended)

Date 09.09.21

Contact Details: Institutions

Funder

To be confirmed

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1. Protocol summary

Title

The effect of increased **P**reterm **A**rginine **INT**ake on biological pathways affecting immune function in infants requiring early parenteral nutrition (PAINT-18)

Population

Preterm infants <29 weeks gestation and/or <1200g

Number of infants

24 infants (completing the study) will be recruited over approximately 12 months

Number of sites

One. Infants will be born at Liverpool Women's Hospital (LWH) or transferred to LWH within 48 hours of birth.

Study duration

Informed consent will take place antenatally, where possible, or within 72 hours of birth. The first study related blood sample will be taken on day 3 of life with the last sample taken on day 30 of life. Other study assessments reflect those routinely performed in preterm infants receiving parenteral nutrition (PN).

Study intervention

All infants will receive standard clinical treatment. 8 infants will receive PN with Primene (AA-P) as the amino acid base, with 8.3% arginine content, and 16 infants will receive PN with additional arginine in the PN bag at a concentration of 18%. These 16 infants will be sub-stratified into two groups based on gestational age (23-26 weeks and 27-29 weeks).

Primary objective

To examine the changes in gene expression present in arginine supplemented infants <29 weeks' gestation and/or <1200g between day 3 and day 30 of life. The changes in gene expression will be compared with those seen between day 3 and day 30 in unsupplemented infants. The genes of interest are those involved in immune function and inflammatory pathways.

Secondary objectives

1. To explore other biological pathways

    i) known to be involved in the pathogenesis of necrotising enterocolitis

    ii) involved in arginine metabolism

    iii) that are related to the insulin-IGF-I axis

2. To determine the changes in metabolomic profiles of these infants during the first 30 days of life.

3. Growth and body composition data during study period.

2. Background

Amino acids (AA) are essential for the development of body structures and for growth. Babies born prematurely often have abnormal development and poor growth. Optimising amino acid intake following preterm birth is essential but understudied. AA can be categorised into essential, non-essential and conditionally essential. Individual AA requirements for preterm infants dependent on parenteral nutrition (PN) are not known except for tyrosine. cysteine, tyrosine, glutamine, arginine, proline, glycine and taurine are regarded as conditionally essential in the preterm infants[1]. Little work has been done on the optimal PN AA formulation for preterm infants, although individual AA deficiency and its potential impact on protein synthesis and growth, is well recognised[1,2]. Arginine plays a vital and versatile role in nutrition and metabolism. Preterm infants have underdeveloped arginine-synthetic pathways and reduced intestinal mass for arginine production[3]. The limited enteral synthetic pathway means arginine is an essential AA for the PN-dependent preterm infant[4,5]. Moreover, it has been suggested that preterm arginine requirements may be higher than healthy term infants. This reflects both increased growth (tissue proteins have a high arginine content - 14% AA[3]) and the central role of arginine in several metabolic pathways[6]. It is unclear how this relates to plasma arginine levels, but these are likely to represent a balance between arginine intake, AA intake and arginine synthesis and the demands of protein synthesis and the multiple metabolic pathways for arginine utilisation[7]. The latter includes providing substrate for nitric oxide (NO) synthase and is essential for ammonia detoxification through the hepatic urea cycle. Very high ammonia levels affect brain function but this is only seen during inborn errors of metabolism. Severe hyperammonaemia results from preterm arginine deficiency and is reversed by arginine supplementation[8-10]. Asymptomatic hyperammonaemia in the preterm infant is relatively common and also responds to supplementation[11]. High ammonia levels (>100µmol/l) are therefore associated with low plasma arginine levels and suggest a functional arginine deficiency rather than simply a biochemical derangement.

Low plasma arginine levels may play a role in immune function[12], necrotising enterocolitis[13,14] (NEC), pulmonary disease[15] and are associated with hyperglycaemia[16]. In animal models, arginine deficiency results in growth failure[17-19]Low plasma arginine levels have been reported in both adults and children with severe sepsis.[20-22] A recent review of serious infection in neonates hypothesised that arginine plays a key role at the interface of the local and systemic immune responses to gut-derived sepsis.[23,24] Arginine depletion has been shown to have effects on T-cell function and immune-signalling may also be affected by the consequent reduction in NO synthesis.[25-27] Low arginine levels result in downregulation of the T-cell receptor *z*-chain in addition to reduced T-cell proliferation and impaired cytokine secretion.[25,26,28] One small randomised-controlled trial of arginine supplementation in preterm neonates has shown a reduction in the incidence of NEC and others have associated hypoarginaemia with NEC.[13,14,23]

Insulin-like growth factor 1 (IGF-I) has also been suggested as a key regulator of neonatal immune responses in maturation processes and inflammation by suppressing proinflammatory Th1 responses.[29] IGF-1 has also been shown to be involved in cord blood T-cell maturation and additionally demonstrates anti-apoptotic effects.[30] Postnatal IGF-1 levels are known to be correlated with neonatal protein and nitrogen balance[31,32] and it is possible there is a mechanism where arginine effects T-cell function directly and also via the IGF-1-insulin axis.

Table 1: Arginine content of parenteral and enteral nutrition

Amino acid source	PN AA usage in UK level 3 NICU	Arginine content (g/100gAA)
Vaminolact (AA-V)	83%	6.3
Primene (AA-P)	15%	8.3
Trophamine (AA-T)	Unlicensed in UK	12.0
Aminoplasmal-Paed (AA-A)	Recently licensed in the UK	9.2
Human milk	-	3.9
Formula		3.8-4.0

Arginine deficiency is common in preterm infants. The combination of reduced synthesis and higher demand leaves the PN-dependent preterm infant vulnerable to arginine deficiency. Table 1 shows that neonatal PN has a higher arginine content than human milk[33] but optimal AA formulation is unknown[34]. We have shown that plasma arginine levels are critically low in very preterm infants receiving PN AA formulations licensed in the UK[35]. Table 2[35] shows that the arginine deficiency remains even after hyperalimentation (optimising total protein/energy intake).

Table 2: Control (C) and hyperalimentation (H) PN groups compared in two RCT: Daily protein and arginine intake and plasma arginine levels

Study	Group	Birthweight (g)	Protein intake (g/kg/day)	Arginine intake (mg/kg/day)	Plasma arginine (micromol/l)
RCT1 (AA-P)	CPN (n=50)	870 (167)	2.6 (0.4)	201 (44)	42 (26-61)*
	HPN (n=52)	858 (166)	3.0 (0.5)	230 (55)	47 (29-75)*
RCT2 (AA-V)	CPN (n=62)	868 (174)	3.0 (0.2)	175 (14)	34 (21-45)**
	HPN (n=45)	917 (157)	3.6 (0.5)	215 (32)	40 (25-53)**

*p=0.19 **p=0.21

Very premature infants are at particularly high risk of early postnatal growth failure[36]. The deficiency of a single AA may undermine strategies to improve growth by impairing protein synthesis. In addition, the current population of PN-dependent preterm infants are a very different population to that originally described with hyperammonaemia responsive to arginine supplementation[11]. Plasma ammonia levels have been related to arginine intake and plasma arginine levels[13,14] in North American studies in very preterm infants. However, the PN formulation (AA-T) used in these studies has an arginine content twice that of current UK formulations (Table 1). The plasma levels of arginine were therefore much higher than reported in infants receiving UK formulations (Table 2). Physiological studies in PN-dependent preterm infants are required to establish the importance of low plasma arginine levels (and therefore potential arginine deficiency) in impaired immune function and suboptimal postnatal metabolic adaptation. Given the urea cycle is a key metabolic pathway affected by arginine deficiency, measuring metabolites that indicate suboptimal urea cycle function may be a useful way of identifying clinically

important arginine deficiency. It is clear plasma ammonia levels are one example of an important metabolic marker of arginine deficiency but metabolomic profiles offer a further novel way to explore a whole range of intermediate metabolites in the urea cycle and other metabolic pathways. This will provide the biochemical markers to allow the PN arginine intake of very preterm intake to be optimised so that deficiency can be avoided.

Metabolomics is the global analysis of endogenous and exogenous metabolites (<1.5kDa) within a biological sample[37] with the potential to study the influence of plasma arginine on metabolism. Metabolomics has been applied to study complications of pregnancy[38] and has shown the importance of another metabolite, myo-inositol, in IGF-1 signalling and growth in children born SGA[39]. Comparing the metabolomes of very preterm infants with different plasma arginine levels would allow a wide range of metabolic pathways to be investigated (for example, urea cycle intermediates) providing data not available from routine biochemical analysis. The metabolomics analysis done by nuclear magnetic resonance (NMR) provides absolute measurements as opposed to mass spectrometry which provides relative values and the Slupsky Lab at the University of California, Davis, is one of few laboratories that have expertise in infant nutrition-related NMR data.

In the first study of this series of physiological studies, (PAINT), we used the technique of microarray and complex bioinformatics analysis to assess the changes in gene expression seen between day 3 and day 10 of life in arginine-supplemented and unsupplemented infants. The analysis of this data has directed us towards important immune and inflammatory pathways that are likely to be changing. This enables us to focus on these pathways in future studies and to utilise multiplex gene expression panels that will focus on identifying changes in immune and inflammatory pathways.

The previous physiological studies our group have undertaken (PAINT and PAINT-NH_4) delivered additional arginine ranging from 12 to 15%, initially as a supplementary infusion and then within the PN bag. We found that even with the 15% arginine supplement many babies still did not achieve adequate plasma arginine levels. The gene expression changes in infants with plasma arginine levels within the normal range compared to infants with low plasma arginine levels exhibited changes in certain immune function pathways similar to the changes seen over the first 10 days of life. This suggests a possible augmentation of the postnatal immune function development in infants with normal (versus low) plasma arginine levels and merits further investigation. Recently, more units within the UK have been changing to Primene as their AA source as it has a longer shelf-life. We therefore need to examine if the changes seen in the previous PAINT study using Vaminolact as the AA source are replicated using Primene as our AA source and additionally whether further increasing the arginine content within the PN bag to 18% can optimise the amino acid profile and result in similar gene expression profile changes. Alongside the gene expression work our group has been looking at the metabolomic profiles in plasma, urine and faeces in these infants in relation to arginine level to help in understanding the metabolic fate and consequences of arginine supplementation. Preliminary analysis of these metabolomics results suggest that those infants that improve their plasma arginine levels after supplementation exhibit different plasma metabolomic profiles than the unsupplemented infants.

Hypotheses

The extent of arginine deficiency is not known in contemporary practice using PN regimens that have been optimised for macronutrients. Further development of PN requires an understanding of the extent and consequences of arginine deficiency. Physiological studies in PN-dependent preterm infants are required to establish the importance of low plasma arginine levels (and therefore potential arginine deficiency) in suboptimal postnatal metabolic adaptation. Given the urea cycle is a key metabolic pathway affected by arginine deficiency, measuring metabolites that indicate suboptimal urea cycle function may be a useful way of identifying clinically important arginine deficiency. Metabolomic profiles offer a novel way to explore a whole range of intermediate metabolites in the urea cycle and other metabolic pathways. The time course of ammonia, arginine and other analytes will indicate when any deficiency and altered metabolism occurs. This will provide the biochemical markers to allow the PN arginine intake of very preterm intake to be optimised so that deficiency can be avoided.

Primary hypothesis: We hypothesise that arginine supplementation will result in changes in gene expression that are consistent with changes in T-cell function and associated inflammatory pathways.

Because the immune/metabolic pathways of interest are clearly defined, the multiplex PCR panel analysis allows the exploration of secondary hypotheses:

1. Arginine supplementation will alter gene expression that is consistent with changes in the inflammatory pathways known to be involved in the pathogenesis of NEC.
2. Arginine supplementation will alter gene expression relevant to metabolic pathways involving arginine metabolism.
3. Arginine supplementation will alter gene expression relevant to metabolic pathways involving the IGF-1-insulin axis.

Further secondary hypotheses in relation to metabolomics are:

4. Arginine supplementation will alter the metabolomics profile for the urea cycle as well as other metabolic pathways in infants on PN during the first 30 days of life

3. Study design

This is a single centre exploratory physiological study. All participants will be managed in accordance with existing local protocols. The study intervention is administering a PN with a higher concentration of arginine and the study will involve the collection of additional blood samples for gene expression and metabolomic analysis, and urine and faeces samples for metabolomic analysis. The period of observation is 30 days and will include collection of all routine clinical data pertinent to the management of nutrition, metabolism and immune function including growth and body composition data.. Eight of the infants in the study will receive standard PN with Primene as the AA base and 16 infants (sub-stratified into two gestational age brackets) will receive the study intervention PN. The comparison of these formulations is considered in Table 3. All babies will have the same blood samples collected.

Table 3: Composition of PN solutions – standard (control) and study intervention PN

Vaminolact	6.3% Arginine [control PN]	18% Arginine [intervention PN]
Ingredient	Volume (mL)	Volume (mL)
Glucose 70%	51	51
Water for Injection	20	39.8
Vaminolact	196	168.5
Sodium chloride 30%	0	0
Sodium glycerol phosphate 21.6%	6	6
Sodium acetate 30%	0	0
Potassium salt	3 (as Potassium Chloride 15%)	1.2 (as Potassium Acetate 49%)
Magnesium sulphate 10%	1.6	1.6
Calcium gluconate 10%	20	20
Peditrace	2.4	2.4
Arginine hydrochloride 20%	0	9.5
Total Volume	**300**	**300**

3.1 Primary endpoint

The pattern of alteration in gene expression between days 3, 10 and 30 in arginine deficient preterm infants after correction of their deficiency by supplementation with arginine. The changes in gene expression will be compared with those seen in unsupplemented infants. The genes of interest are those involved in T-cell function and associated inflammatory pathways.

3.2 Secondary endpoints

Secondary endpoints include:

a) To explore biological pathways known to be associated with NEC.
b) To explore biological pathways known to be involved in arginine metabolism.
c) To explore biological pathways that are related to the IGF-1-insulin axis.
d) Whether intermediates in arginine metabolism vary according to ammonia and arginine levels and arginine supplementation.
e) The difference in blood ammonia and plasma arginine levels between days 3, 10 and 30 and the difference in metabolomic profiling particularly in relation to urea cycle intermediates.
f) To assess if there is a link between low plasma arginine levels and other metabolic pathways that we have not anticipated in the original hypothesis.
g) To explore growth and body composition changes during the study period.

3.3 Study endpoint

The final study endpoint, after which the study will be closed, is the completion of analysis of study-related data.

4. Study population

4.1 Inclusion criteria

Infants born <29 weeks' gestation and/or with birthweight <1200g and who are admitted to the Neonatal Unit at Liverpool Women's Hospital within 48 hours of birth.

4.2 Exclusion criteria

a) Infants who are unlikely to survive the first week after birth.

b) Infants known (or suspected to have) a diagnosis of inborn error of metabolism or serious liver dysfunction

c) Parents who are unable to give informed consent

5. Enrolment and recruitment

5.1 Eligibility and consent

Eligible patients will be identified by the clinical team and highlighted to the research team. The parent/guardian(s) of each potentially eligible patient will be approached (ideally both parents/guardians if appropriate) antenatally, where possible, or postnatally when the baby has been stabilised on the neonatal unit. The Investigator will explain the study fully to the patient's parent(s)/guardian(s) using the Patient Information Leaflet. The parent/guardian(s) will be able to ask the investigator questions to clarify what the study involves. They will then be given some time (minimum 1 hour) to discuss the information without the investigator present. The parent/guardian(s) will be requested to write any further questions down on the back of the information sheet. The investigator will then return and request written consent if the parents are willing for their infant to take part and the investigator is satisfied the parents understand the study. If more time is requested then the study information can be considered for a period up to 72 hours from birth. The Informed Consent Form will be signed and personally dated by both a parent/guardian and the investigator. A copy of the signed form will be provided to the parent(s)/guardian(s), another copy will be placed in the patient record, and the original retained with the research record. Nursing and medical staff may be involved in describing clinical aspects of the study to parent(s)/guardian(s), particularly where clarification of routine clinical management of PN delivery is required.

5.2 Patient treatment and management

The nature of this study will be explained as part of the consent process. The exact additional quantities of blood required for research purposes will be explained, as well as the process for collecting the blood, urine and stool samples. All additional blood sampling will be taken at the same time as routine clinical samples so as not to increase the number of episodes of blood sampling.

6 Assessments and procedures

6.1 Study schedule

Consent will be sought within 72 hours of birth. Following consent, all required study data for the period since birth will then be entered on the appropriate CRF including the growth measurements at birth. The study schedule is summarised in Table 4. The process of collecting large amounts of routine monitoring data has been evaluated and refined in a previous study.

Table 4: Study schedule for the PAINT-18 study

Day	Study	Nutrient and infusion data	Study PN	Plasma AA levels	Blood for gene expression	Metabolomics
1	Consent	•	•*			
2	Consent	•	•*			
3	Consent	•	•	•	•	•
4		•	•			
5		•	•			
6		•	•			
7		•	•			
8		•	•			
9		•	•			
10		•	•	•	•	•
11		•	•			
12		•	•			
13		•	•			
14		•	•			
15		•				
16		•				
17		•				
18		•				
19		•				
20		•				
21		•				
22		•				
23		•				
24		•				
25		•				
26		•				
27		•				

28		•				
29		•				
30	End	•		•	•	•

* Study PN will be administered from time of consent (which may mean from Day 1 of life if antenatally recruited) and will stop on Day 14 or earlier if adequate enteral feeds achieved earlier

6.1.1 Intravenous/enteral nutrition, fluid and drug infusion data

The hourly volume of each component of the intravenous/enteral nutrition, fluid and drug infusions is captured on routine nursing charts and will be transferred onto a daily paper CRF. Each 24 hour period will start at the time of birth and data will be collected for 30 completed days after birth.

6.1.2 Biochemical/nutritional monitoring

Biochemical and nutritional monitoring will follow the protocol outlined in the LWH NICU PN guidelines. This will include routine growth measurements and body composition data measured by electrical impedance (Bioscan Touch i8 Nano, Maltron Ltd). Data will be collected from the electronic patient record and recorded on the appropriate paper CRF for 30 days.

6.1.3 Infection monitoring

Monitoring for infection will follow the protocol outlined in the LWH NICU guidelines for infection. Daily CRP, white cell count (and neutrophils) and platelet data will be recorded in medical record and transcribed to the appropriate CRF for 30 days from birth. Blood culture results will be recorded on the day of sample in the medical record and will be transcribed onto the CRF until day 30.

6.2 Blood sampling and processing

PN blood tests: Routine biochemical monitoring will take place in accordance with LWH PN guidelines. An additional plasma AA level (0.2ml) will be collected on day 30 of life. All routine blood samples will be processed according to standard practice and sent to the laboratories at the Alder Hey Children's Hospital. These results will be entered into the medical record. Results will be transcribed onto the CRF.

6.2.1 Blood ammonia analysis

Blood ammonia levels (blood spot) will be performed using point of care (POC) testing device (PocketChem BA analyser) on days 3, 10 and 30. The timing of individual samples may be adjusted by $\pm$1 day if clinically indicated sampling (eg timing of AA sampling) dictates this is desirable. Blood ammonia levels >100µmol/l will require repeat testing and then laboratory verification (and clinical advice from Alder Hey Children's Hospital) if confirmed.

6.2.2 Blood for RNA extraction for gene expression analysis

Blood samples (0.2ml) will be taken on days 3, 10 and 30 of life for RNA extraction for gene expression analysis. The sample will be collected in conjunction with routine clinical AA levels. The timing of individual samples may be adjusted by ±1 day if clinically indicated sampling (eg timing of AA sampling) dictates this is desirable. Samples will be processed and stored at Liverpool Women's Hospital.

6.2.3 Metabolomics

A blood sample for metabolomics analysis will be collected on days 3, 10 and 30 of life, at the same time as the other study bloods, with a total blood sample volume of 3.45 ml/kg (split between all study samples and the three timepoints). The timing of individual samples may be adjusted by ±1 day if clinically indicated sampling (e.g. timing of AA sampling) dictates this is desirable. Where possible (depending upon availability) urine and faeces samples will be collected on the same days. Samples will be stored and transported as a batch for analysis at Department of Nutrition laboratories (University of California, Davis, USA).

6.3 Study PN containing 18% arginine

The infants will receive study PN from the time of their recruitment until day 14 of life (most infants will be on mainly enteral milk feeds by this time). The amount of arginine delivered will not exceed that which has been used in a previous study of arginine supplementation and for which there were no reported side effects or safety concerns.

7 Safety and adverse event reporting

7.1 Safety assessments

Adverse events are relatively common in this patient group due to immaturity and to concomitant disease processes. Routine clinical monitoring will be used to ensure that biochemical monitoring stays within limits defined within LWH clinical guidelines. Abnormal amino acid profiles are discussed with a biochemist. The levels of PN macronutrients are defined in standard protocols and have been used in several previous studies without safety concerns. Should an adverse event occur in a baby receiving the additional arginine infusion the details will be referred to a consultant neonatologist to act as an independent clinical assessor.

7.2 Recording safety information

High blood ammonia (>280µmol/l) can be a sign of rare inborn errors of metabolism. Given blood ammonia levels will be available from POC within minutes of testing there will be a SOP to follow for blood ammonia levels >100µmol/l to ensure appropriate clinical biochemical advice at the Alder Hey Children's Hospital is obtained and further investigations are performed if required.

8 Ethical Considerations

The study will abide by the principles of the World Medical Association Declaration of Helsinki (1964) and subsequent amendments. The study will be conducted according to ICH Good Clinical Practice and the NHS Research Governance Framework.

8.1 Main ethical considerations

a) Time at which consent is obtained. Clearly, obtaining informed consent antenatally or within the first 72 hours of life from parents whose child will/has been born very prematurely requires experienced clinicians/nurse specialists who are experienced at imparting information to parents in times of extreme stress. It is expected that consent will be obtained by the dedicated clinical research fellow in all but exceptional circumstances. In these exceptional circumstances, informed consent will be obtained by the chief investigator or other experienced clinical/research worker specifically trained to take consent for this study. It will be clearly explained to parents that they may remove their child from the study at any time.

b) Informed consent from a neonatal population. The parent or legal representative of the child will have an interview with the investigator (see above) during which they will be given time to understand the objectives, risks and inconveniences of the study and the conditions under which it is to be conducted. They will be provided with written information and contact details of the local study personnel should they require additional information.

c) Additional blood sampling (0.05mL for ammonia blood spot, 0.2 mL for gene expression analysis, 0.6-1.2mL for metabolomics (dependent on baby's weight). Total study specific blood sampling will be equivalent to 3.45mL/kg over 30 days. There is no reason to think that this extent of blood causes any harm in the study population. Heidmets et al found that sampling 2.3 mL / kg over 12 hours during a pharmacokinetic study in this population did not have any effects on haemodynamic parameters or transfusion requirements.[40] The European Medicines Agency guidelines for blood sampling suggest that reasonable limits for sampling are 2.4 mL/kg per 28 days.[41]

d) Routine blood sampling. Most blood tests required for this study are taken in the course of routine clinical practice. While this study does require extra blood to be sampled, extra sampling episodes will be avoided by simultaneous study and routine blood sampling.

e) Additional arginine content in PN. The arginine has been stability tested both within the PN bag and alongside other infusions to ensure compatibility with other routine neonatal infusions. Our previous work has delivered up to 15% arginine with no concerns of side-effects. There is no evidence that arginine supplementation at this level confers any safety concerns. A published study in neonates delivered arginine of 20mg/kg/hour and did not report any adverse effects associated with that level of arginine supplementation.[13]

f) Additional samples in the form of urine and faeces will be collected from the infants. This will be analysed for metabolomics. Collecting these samples will be at times of routine cares and will have no additional impact on the infant.

8.2 Ethical approval

Ethical approval will be sought from the Local Research Ethics Committee (LREC) via the IRAS application system.

8.3 Informed consent

This process is outline in section 5.1

9 Statistical analysis

9.1 Sample size

A sample size of 24 is based on similar scale physiological studies that indicate that this sample size provides sufficient information on alterations in gene expression and amino acid metabolism in very preterm infants.

9.2 Primary analysis

The primary outcome of the study is looking at gene expression in infants receiving increased arginine content. The control group of patients will enable the changes in gene expression to be interpreted by excluding confounders, such as routine changes in gene expression in the first 30 days of life.

9.3 Secondary outcomes

Alterations in gene expression between days 3, 10 and 30 of life in the same patients will be explored and alterations in gene expression between those babies with low and normal arginine levels. Primary and secondary analyses will be further interpreted using the accompanying nutritional intake data (arginine, protein and energy intake).

The secondary outcomes of the study related to metabolomics will be assessed by stratifying infants according to plasma arginine level and postnatal age to allow variation in metabolomics profiles to be explored (e.g. comparing the urea cycle intermediates between the 1st and 4th quartiles of plasma arginine levels and also between days 3, 10 and 30).

10 Study Monitoring

10.1 Risk Assessment

In accordance with The Sponsor's requirements this study will undergo a risk assessment completed in partnership between LWH R&D department and the Principal Investigator. In conducting this risk assessment, the contributors consider potential patient, organisational and study hazards, the likelihood of their occurrence and resulting impact should they occur.

10.2 Source documents

The source documents will be either the medical record or the case record forms (CRFs). The medical record has several components:

a) written records made at the bedside by nursing staff (medication administration, fluid administration);

b) written records made by medical staff or advanced neonatal nurse practitioners (prescriptions for medication and PN);

c) the unit patient data management system (BadgerNet) (all other observations made by the clinical team including the results of laboratory and imaging investigations);

The source data for the study will be held in the medical record with the exception of the following pieces of information for which the CRF will be the source document:

i) Blood ammonia levels

ii) Metabolomic profiles

iii) Gene expression data

The date(s) of conducting the informed consent process including the date of provision of parent information, date of written consent and the fact that the patient is participating in a research study will be added to the patient's medical record chronologically.

10.3 Data capture methods

A paper case report form is the primary data collection instrument for the study. The CRF will have several components. All data requested on the CRF will be recorded. All missing data will be explained. If a space on the CRF is left blank, N/D will be entered. If the item is not applicable to this case then N/A will be entered. All entries will be printed legibly in black ink. If any entry error has been made, to correct such an error, a single line will be drawn through the incorrect entry and the correct data entered above it. All such changes will be initialled and dated. Errors will not be erased (or whited out).

Data will be transferred to a database using a front-end designed to minimise data entry error.

10.4 Site monitoring by the sponsor

The R&D Department, Liverpool Women's Foundation Trust will receive copies of the patient consent form within one week and regular checks of consent records in patient notes will be made. CRF data will be checked for adherence to the study protocol, missing or unusual values (range checks) and consistency within participants over time. Discrepancies that have been raised will be queried.

The Principal Investigator will review rates of recruitment, missing outcome data, SAEs, ADR, study withdrawals. The Sponsor's Standard Operating Procedures for protocol breaches and urgent safety measures will be adhered to.

10.5 Confidentiality

Individual participant medical information obtained as a result of this study is considered confidential and disclosure to third parties is prohibited with the exceptions noted below. Case report forms (CRF) will be labelled with the study identification number. Medical information may be given to the participant's medical team and all appropriate medical personnel responsible for the participant's welfare.

10.6 Quality assurance, quality control and audit

Quality assurance for the study will be provided by the R&D Department at LWFT in line with its policies and SOPs. Where independent audit is required this will be commissioned by the R&D Department, Liverpool Women's Foundation Trust.

10.7 Records retention

The Chief Investigator will make arrangements to store the essential study documents including the Investigator Site File for 20 years. LWFT will be responsible for archiving the medical record. The R&D Department at LWFT will store completed CRFs for the same period.

10.8 Data governance

Data collected from the babies involved in the study will be anonymised and thereafter shared with collaborators at the University of California for the laboratory analysis work of the metabolomics. This would mean that anonymised data of the participants of the study will leave the European Union (EU).

11 Regulatory approval

The study will be submitted for ethical approval via IRAS. All further substantial amendments will be submitted to the LREC. We have considered carefully whether this study is a Clinical Trial of an Investigational Medicinal Product (CTIMP). The purpose of the study is to examine the effects of arginine on gene expression. We are not examining the clinical efficacy or the safety of arginine or its pharmacological effects or disposition. Having consulted the MHRA's algorithm (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/317952/Algothrim.pdf) we believe that this study is a study about the physiological effects of arginine and that this study is not a CTIMP. This has been confirmed in an email from the MHRA.

12 Indemnity

The co-sponsor Liverpool Women's Hospital NHS Foundation Trust has insurance coverage for liabilities relating to harm caused by negligence in the design or management of the study under the terms of the NHS Clinical Negligence Scheme for Trusts. The sponsor does not provide cover for liabilities relating to non-negligent harm.

13 Publication policy

Primary responsibility for preparing publications for the study will lie with the Principal Investigator. It is expected that all other investigators will contribute at all stages of project design, execution, analysis and interpretation so

that all investigators named in section 1 will be authors on all publications relating to the aspects of the project with which they are associated. This does not preclude other authors from being involved.

14. Amendments

Proposed amendments to version 1.0 (changes in red in the protocol):

1. Change of PI from Dr Laura Burgess (now co-investigator) to Dr Frances Callaghan

2. Addition of new co-investigators: Diane McCarter, Keziah Davies and Lauren Williams (the latter replaces Sarah Murray)

3. Parenteral amino acid source changed back to Vaminolact (to be consistent with previous PAINT and PAINTNH4 studies): Table 3

4. Growth and body composition measurements specified as a secondary outcome (sections 1, 3.2 (g) and 6.1.2)

15. References

1. te Braake FWJ, van den Akker CHP, Riedijk MA et al. Parenteral amino acid and energy administration to premature infants in early life. *Sem Fetal Neonatal Med* 2007;**12**:11-18.

2. Mayes K, Tan M, Morgan C. Effect of hyperalimentation and insulin-treated hyperglycaemia on tyrosine levels in very preterm infants *JPEN* 2014;**38**:92-8.

3. Wu G, Jaeger LA, Bazer FW, Rhoads M. Arginine deficiency in preterm infants: biochemical mechanisms and nutritional implications J Nutr Biochem 2004;15:442-451.

4. Burrin DG, Davis TA. Protein and amino acids in enteral nutrition *Curr Opin Clin Nutr Metab Care* 2004;**7**:79-87

5. Brunton JA, Bertolo RF, Pencharz PB, Ball RO. Proline ameliorates arginine deficiency during enteral but not parenteral feeding in neonatal piglets. *Am J Physiol* 1999;**277**:E223-31.

6. Klein CJ. Nutrient requirements for preterm formulas. A report from the American Society for Nutritional Sciences, Life Sciences Research Office *J Nutr* 2002;**132**:1431S-49S

7. Wu G, Bazer FW, Davis TA et al Arginine metabolism in development, health and disease. *Amino Acids* 2009;**37**:153-68.

8. Heird W, Nicholson J Driscoll J Hyperammonaemia resulting from intravenous alimentation using a synthetic l-amino acids: a preliminary report *J Pediatr* 1972;**81**:162-5.

9. Shohat M, Wielunsky E, Reisner SH.Plasma ammonia levels in preterm infants receiving parenteral nutrition with crystalline L-amino acids. *JPEN J Parenter Enteral Nutr*. 1984;**8**:178-80.

10. Thomas DW, Sinatra FR, Hack SL, Smith TM, Platzker AC, Merritt RJ. Hyperammonemia in neonates receiving intravenous nutrition. JPEN J Parenter Enteral Nutr. 1982;**6**:503-6.

11. Batshaw ML, Wachtel RC, Thomas GH, Starrett A, Brusilow SW. Arginine-responsive asymptomatic hyperammonemia in the premature infant. J Pediatr. 1984;**105**:86-91.

12. Envoy D, Lieberman M, Fahey T, Daly I. Immunonutrition: the role of arginine *Nutrition* 1998;**14**:611-17.

13. Amin H, Zamora S, McMillan D et al. Arginine supplementation prevents necrotising enterocolitis in the premature infant *J Pediatr* 2002;**140**:425-31.

14. Zamora SA, Amin HJ, McMillan DD et al. Plasma L-arginine concentrations in premature infants with necrotising enterocolitis. *J Pediatr* 1997;**131**:226-32.

15. Castillo L, DeRojas-Walker T, Yu YM et al. Whole body arginine metabolism and nitric oxide synthesis in newborns with persistent pulmonary hypertension. *Pediatr Res* 1995;**38**:17-24.

16. Burgess L, Morgan C, Mayes K, Tan M. Low plasma arginine levels and blood glucose control in very preterm infants receiving two different parenteral nutrition regimens *JPEN* 2014;**38**:243-53.

17. Wu G, Knabe DA, Kim SW. Arginine nutrition in neonatal pigs. *J Nutr* 2004;**134**:S2783-90.

18. Kim SW, McPherson RL, Wu G. Dietary arginine supplementation enhances the growth of milk-fed young pigs *J Nutr* 2004:**134:**625-30.

19 . Tan B, Yin Y, Liu Z et al. Dietary L-arginine supplementation increases muscle gain and reduces body fat mass in growing-finishing pigs. *Amino Acids* 2009;**37**:169-75.

20. Freund H, Atamian S, Holroyde J, et al: Plasma amino acids as predictors of the severity and outcome of sepsis. *Ann Surg* 1979; 190:571-576

21. Luiking YC, Steens L, Poeze M, et al: Low plasma arginine concentration in septic patients is related to diminished *de novo* arginine production from citrulline. *Clin Nutr* 2003; 22 (Suppl 1):S26

22. Weiss SL, Haymond S, Ralay Ranaivo H, Wang D, De Jesus VR, et al. (2012) Evaluation of asymmetric dimethylarginine, arginine, and carnitine metabolism in pediatric sepsis. Pediatr Crit Care Med 13: e210–218

23. Polycarpou E, Zachaki S, Tsolia M, et al. Enteral L-arginine supplementation for prevention of necrotizing enterocolitis in very low birth weight neonates: a double-blind randomized pilot study of efficacy and safety. JPEN *J Parenter Enteral Nutr* 2013;**37**:617–22.

24. Badurdeen S, Mulongo M, Berkley JA. Arginine depletion increases susceptibility to serious infections in preterm newborns. *Pediatr Res.* 2015 Feb;**77**(2):290-7

25. Munder M, Schneider H, Luckner C, et al. Suppression of T-cell functions by human granulocyte arginase. *Blood* 2006;**108**:1627–34.

26. Munder M. Arginase: an emerging key player in the mammalian immune system. *Br J Pharmacol* 2009;**158**:638–51.

27. Diefenbach A. Requirement for Type 2 NO Synthase for IL-12 Signaling in Innate Immunity. *Science* 1999;**284**:951–5.

28. Kropf P, Baud D, Marshall SE, et al. Arginase activity mediates reversible T cell hyporesponsiveness in human pregnancy. *Eur J Immunol* 2007;**37**:935–45.

29. Puzik A, Rupp J, Troger B, Gopel W, Herting E, et al. (2012) Insulin-like growth factor-I regulates the neonatal immune response in infection and maturation by suppression of IFN-gamma. *Cytokine* 2012;**60**(2):369-76

30. Law HK, Tu W, Liu E, Lau YL. Insulin-like growth factor I promotes cord blood T cell maturation through monocytes and inhibits their apoptosis in part through interleukin-6. *BMC Immunol*. 2008;**9**:74

31. Yeung MY, Smyth JP Nutritionally regulated hormonal factors in prolonged postnatal growth retardation and its associated adverse neurodevelopmental outcome in extreme prematurity. *Biol Neonate* 2003;**84**:1-23.

32. Engstrom E, Niklasson A, Wikland KA, Ewald U, Hellstrom A. The role of maternal factors, postnatal nutrition, weight gain and gender in regulation of serum IGF-I among preterm infants *Pediatr Res* 2005;**57**:605-10.

33. Rassin DK Shattuck KE. Enteral amino acid and protein digestion, absorption and metabolism. In: Neonatal Nutrition and Metabolism Thureen PJ and Hay WW (2nd Edition) Cambridge University Press, Cambridge, 2005, p332-9.

34. Morgan C. Early amino acid administration in very preterm infants: too little too late or too much too soon? *Sem Fetal Neonatal Med* 2013;**18**:160-5.

35. Morgan C, McGowan P, Burgess L, Mayes K, Tan M. Hyperalimentation using current UK parenteral amino acid formulations does not prevent low plasma arginine levels in preterm infants. *Arch Dis Child* 2014;**99(Suppl1)**:A30.

36. Morgan C, McGowan P, Herwitker S et al. Postnatal head growth in preterm infants: a randomised controlled parenteral nutrition study. *Pediatrics*2014;**133**:e120-8.

37. Dunn WB, Broadhurst DI, Atherton HJ, Goodacre R, Griffin JL. Systems level studies of mammalian metabolomes: the roles of mass spectrometry and nuclear magnetic resonance spectroscopy. *Chem Soc Rev*. 2011;**40**:387-426.

38. Heazell AE, Bernatavicius G, Warrander L, Brown MC, Dunn WB. A metabolomic approach identifies differences in maternal serum in third trimester pregnancies that end in poor perinatal outcome. *Reprod Sci.* 2012;19:863-75.

39. Stevens A, Bonshek C, Whatmore A et al. Insights into the pathophysiology of catch-up compared with non-catch-up growth in children born small for gestational age: an integrated analysis of metabolic and transcriptomic data. P*harmacogenomics J.* 2014;**14**:376-84.

40. Heidmets LT, Metsvaht T, Ilmoja ML, Pisarev H, Oselin K, Lutsar I. Blood loss related to participation in pharmacokinetic study in preterm neonates. *Neonatology.* 2011;**100**:111-5.

41. CHMP (Committee for Medicinal Products for Human Use) & PDCO (Paediatric Committee). Guideline on the Investigation of Medicinal Products in the Term and Preterm Neonate. *European Medicines Agency*. 25 June 2009; Doc. Ref. EMEA/536810/2008.