The effect of higher protein human milk fortifier on growth in preterm infants

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This thesis is submitted for the degree of Doctor of Philosophy

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THE UNIVERSITY of ADELAIDE

May 2014

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Abbreviations of commonly used words and units of measurement

ABS	Australian Bureau of Statistics
ADP	Air-displacement plethysmography
ANZCTR	Australian and New Zealand Clinical Trials Registry
ANZNN	Australian and New Zealand Neonatal Network
BIS	Bioelectrical Impendence Spectroscopy
BUN	Blood Urea Nitrogen
BW	Birth Weight
CA	Corrected Age
CI	Confidence Interval
CIS	Clinical Information Services
CONSORT	Consolidated Standard of Reporting Trials
CPAP	Constant Positive Airway Pressure
CRF	Case Report Form
CT	Computed Tomography
DHA	Docosahexaenoic acid
DMAC	Data Management and Analysis Centre
DXA	Dual Energy X-ray Absorptiometry
EBM	Expressed Breast Milk
ECW	Extracellular Water
EDD	Estimated Delivery Date

FFM	Fat Free Mass
FM	Fat Mass
FMC	Flinders Medical Centre
g	Grams
g/kg/day	Grams per kilogram of body weight per day
GA	Gestational Age
HFOV	High Frequency Oscillation Ventilation
HHFNC	Humidified High Flow Nasal Cannula therapy
HMF	Human Milk Fortifier
ICW	Intracellular Water
IQ	Intelligence Quotient
IPPV	Intermittent Positive Pressure Ventilation
IVF	In-Vitro Fertilisation
IVH	Intraventricular Haemorrhage
LCPUFA	Long Chain Polyunsaturated Fatty Acids
LM	Lyell McEwin Hospital
MDI	Motor Development Indices
ml	Millilitre
MRI	Magnetic Resonance Imaging
MeSH	Medical Subject Heading
ml/kg/day	Millilitres per kilogram of body weight per day
mm/d	Millimetres per day
NEC	Necrotising Enterocolitis
NED	Neonatal Early Discharge

NICU	Neonatal Intensive Care Unit
PDI	Psychomotor Development Indices
Poppet	Providing Optimal Protein for Prems via Enteral Tubes
RCT	Randomised Controlled Trial
RR	Relative Risk
SCBU	Special Care Baby Unit
SD	Standard Deviation
SGA	Small for Gestational Age
SOP	Standard Operating Procedure
TBW	Total Body Water
TGA	Therapeutic Goods Administration
UR	Unit Registration Number
VLBW	Very Low Birth Weight
WCH	Women's and Children's Hospital
WMD	Weighed Mean Difference

Abstract

Preterm infants are difficult to adequately nourish due to their immature organ systems and prematurity related illnesses. Human milk is accepted as the preferred feed for the preterm infant but needs to be supplemented with protein, carbohydrate, vitamins and minerals to meet the metabolic needs of the infant. Currently available commercial fortifiers do not meet the recommended protein intakes suggested in the literature. This thesis tested the hypothesis that preterm infants fed a human milk fortifier with a protein content of 1.8 grams protein per 100 ml expressed breast milk would experience greater weight gain than infants fed the current nursery practice amount – 1.0 grams protein per 100 ml expressed breast milk.

Criteria for eligibility of infants in this study were birth at 28–32 weeks gestation and a planned breast milk diet. Power analysis indicated that to detect a clinically significant weight gain increase of 3.31 grams per day, 60 infants in total would be required. After parental consent was obtained, 60 infants were randomised into the study between February 2012 and February 2013, with 31 in the High protein group and 29 in the Standard group. Multiple births were randomised as individual infants. Infants in the High protein group received a human milk fortifier (FM-85, Nestle) enriched with Protifar (Nutricia) which provided 1.8 grams protein per 100 ml expressed breast milk. Infants in the Standard group received a human milk fortifier (FM-85) that was equivalent to standard care and provided 1 gram protein per 100 ml expressed breast milk. The Standard diet was made isocaloric to the High Protein by the addition of carbohydrate (PolyJoule, Nutricia). The study period for infants began at randomisation and ceased when the naso-gastric tube

was removed. Infants were weighed daily by care staff and weekly by trained research personnel. Length and head circumference – important measures of growth – were assessed weekly. Lean mass gain, which is better associated with adult metabolic health outcomes than adipose tissue gain in the in-hospital period, was also assessed weekly. Blood and urine chemistry markers were assessed weekly and every two weeks, respectively, as an assessment of protein nutritional status. A weekly sample of breast milk was collected if supply was abundant. Lean mass was assessed using Bioelectrical Impedance Spectroscopy, which was validated for use in preterm infants as part of this thesis. Breast milk was assessed for protein content to ensure that intake calculations were based on true, not assumed values.

There were no differences in the primary outcome of weight gain or the secondary outcomes of length gain, head circumference gain or small for gestational age at discharge status. There was a significant trend for increased lean mass as a percentage of body weight in the high protein infants ($p=0.03$). Blood urea nitrogen and urine urea measurements were significantly higher in the High protein infants. Base excess measurements were significantly decreased in High protein infants however no infants experienced metabolic acidosis.

Increasing the protein content of human milk fortifier to 1.8 grams protein did not increase weigh gain, length gain or head circumference gain in preterm infants born at 28–32 weeks gestation. While the intervention was well tolerated it is the conclusion of this thesis that the protein content of human milk fortifier does not need to be increased to 1.8 grams protein per 100 ml expressed breast milk. Further studies are required to determine the optimal macronutrient content of human milk fortifier to improve preterm growth.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Jessica Reid

Acknowledgements

Thank you to all the mums and dads for agreeing to be involved in research to help others families. Your generosity and empathy at such a difficult time is awe-inspiring and a reminder to me to keep working to achieve better outcomes for all babies. To your beautiful babies, thanks for letting a rookie measure you, feed you, change your nappies and dress you in new outfits. You guys are the hardest fighters I know.

I acknowledge the work of statistical legends, Mr Tom Sullivan and Dr Jennie Louise from Data Management Analytical Services, University of Adelaide, who greatly assisted me in writing the technical aspects of the Data Analysis Plan for the Poppet Study and performed the part of the statistical analyses.

I acknowledge the expertise of Mr Lou Nesci and Mrs Rosemarie Gerace at SA Pathology who analysed the blood and urine metabolites. I thank Mr Enzo Raneri and Dr Michael Metz who provided expert opinion on the meaning of these data.

Many, many thanks are owed to Mrs Louise Goodchild and Ms Ros Lontis, the neonatal research nurses at WCH. Their dedication and professionalism is to be admired and they are directly responsible for the success of this project and all neonatal research projects that are undertaken at WCH. I am eternally grateful for all the support, teaching and patience they bestowed on a first time researcher.

I acknowledge the highly skilled work undertaken by the neonatal Clinical Information Service, particularly that of Mrs Ann Fitzgerald and Mrs Cindy Golding who allowed me to access their neonatal database in order to compile my own.

Thank you to Mrs Jane Raymond, Head of Dietetics and Food Services and Mrs June Bailey, Manager of Food Services, who were invaluable not only in the months of preparation but also on all of the 440 days that the study ran. Mrs June Bailey generously rearranged her team's daily processes in order to accommodate the Poppet Study without which the study could not have been performed in the intended manner. The Food Services team is passionate, professional and highly skilled thanks to your leadership and guidance. I thank them for their ability to problem solve, to accommodate my dozens of last minute order changes and their constant cheerfulness. It was an absolute pleasure to work with all of you.

To Mrs Pauline Lowe, Mrs Julie Rundell, Ms Lynne Headland and all of the neonatal nurses and midwifes of WCH and LM, thank you for your support of all neonatal research. The continual improvement of care for neonates is due to you. Thank you for all the assistance and enthusiasm you showed for Poppet. The care and love you show the babies and their families for their stay in the nursery is to be admired and celebrated. Thank you for all the hard work you do every day for them.

To Wendy, who was Miss Helbers when we started and is now Mrs Hackett plus Lily, I loved being in the nursery with you. You were a fantastic and patient teacher and I am grateful for all of your work on the BIS Validation Study. I would like to thank Associate Professor Leigh Ward for teaching me the lab techniques necessary for the BIS Validation Study and for his meticulous review of the accompanying chapter.

I acknowledge Nestle Infant Nutrition, who through Peter Fryer, Science Manager, donated the Pre NAN FM 85 Human Milk Fortifier used in the trial. I would also like to thank Nutricia who donated Protifier and PolyJoule. Nestle and Nutricia were

not involved in study conception, study design or the preparation of any manuscript associated with this project. Thank you to the WCH Foundation who supported this project.

To Dr Chad Andersen and your clinical team, I have been spoilt forever by starting my career in such a research supportive nursery. Some of the best neonatal research in the world comes out of this nursery and I am grateful that you allowed me to contribute to that. Thank you for making me feel so welcome.

Thank you to Associate Professor Michael Stark, who acted as an Investigator on the Poppet Study and provided immense support to me at a nursery and academic level. Your dedication and enthusiasm for neonatal research is something for me to aspire to and I thank you for all your valuable insight.

Thank you to Dr Andy McPhee, my best cheerleader. Your understanding of preterm infant nutrition is world class and it has been an honour to be supervised by you. Thank you for always finding time for me and for your innate kindness.

To Professor Maria Makrides, it has been an honour and a pleasure to graduate from your Genius Factory. Thank you for all your guidance and support over this PhD. Your work ethic to improve maternal-paediatric nutrition is awe-inspiring. To have worked so closely with such an accomplished woman in science is something I will be forever proud of.

To Dr Carmel Collins, who was my ally throughout this marathon, thank you for the amount of time that you were able to spend with me while running your own international clinical trial. Your ability to simplify even the most complex of ideas is second to none and your endless patience is a true testament to your character.

To Dr Mum and Dr Dad, this belongs to you as much as it does me. Thank you for the genes, words, support and love I needed to make it out the other side of this adventure.

Literature review

Chapter 1 Literature review

An infant born before the completion of 37 weeks gestation is classified as preterm. There is some conjecture about the earliest gestation age (GA) an infant can be born and survive, though the most viable are above 22-23 completed weeks (ANZNN 2013). Birth weight and GA correlate with expected outcomes for preterm infants, such that more mature infants (born between 34 and 37 weeks GA) are healthier and have better long term outcomes than more immature infants. The more immature preterm infants, referred to as 'very preterm', form the focus of this thesis.

The very preterm infant is underdeveloped and often ill-equipped for life outside the mother. Organs are not fully formed and the infant requires a high level of care in the early neonatal period. Effective respiratory support and cardiac health care is vital to the survival of the very preterm infant and the developments in these fields of neonatal care have greatly increased infant survival. This increased survival leads to the challenge of adequately nourishing very preterm infants, who have a high nutritional requirement but underdeveloped organs.

The importance of good quality nutrition and growth for very preterm infants has emerged in recent decades as necessary for good long-term health. Poor nutrition, both quantity and quality, in the first months of life can have adverse effects on child and adult neurodevelopmental and metabolic health. Good quality growth – gaining lean tissue in preference to fat mass – is the favoured outcome in the nutritional management of preterm infants. Evidence suggests that lean mass accretion is dependent on protein intake. Recently, established techniques allow

for the body composition of an individual infant to be assessed which is vital in ensuring neonatal growth is optimal, for immediate and future health.

The aim of this literature review is to examine the current knowledge regarding nutritional management and growth of preterm infants in particular with respect to enteral nutrition in human milk fed infants and effect on growth and body composition.

1.1 The preterm infant

1.1.1 Incidence of preterm birth is increasing

Preterm births are increasing in the western world for a number of reasons. More women are delaying childbirth into their 30s and 40s and more couples are using assisted reproduction techniques. The incidence of multiple birth is growing with both increasing maternal age and the use of these techniques. Increased fetal monitoring also means that obstetricians are increasingly inducing birth before gestation is complete if the fetus is known to be distressed (Loftin et al. 2010). These situations independently increase the risk of preterm birth and often compound to further increase risk. The rate of preterm birth in Australia is around 8% (ABS 2006).

1.1.2 Consequences of preterm birth

Advances in neonatal medicine have been rapid. Australian data shows that in 1900, 103 children per 1000 live births were expected to die before reaching one year of age. By 2000 that number had dropped to 5.2 (ABS 2006). Not surprisingly

the most immature infants have the poorest chances of survival. Infants born less than 24 completed weeks have a 53% chance of survival at discharge, while infants born at a gestational age of 26 weeks experience an increased survival rate of 83% (ANZNN 2013). Greater fetal maturity at birth has improved survival with rates of 94% and 98% for infants born at 28 and 30 completed weeks, respectively (ANZNN 2013).

The estimated cost of caring for a preterm infant in an Australian neonatal intensive care unit is $2 500 per day (adjusted for inflation to 2013 figure using Reserve Bank of Australia Inflation Calculator (Crump et al. 2011a) on 1997 figures published by Doyle and colleagues (2005)). For infants that are born before 26 weeks GA, the cost from birth to discharge is estimated at $139 000 (adjusted for inflation to 2013 figure using Reserve Bank of Australia Inflation Calculator (Crump et al. 2011a) on 1997 figures published by Doyle and Colleagues (2005)). There are also significant costs related to ongoing medical, social and educational expenses that do not apply to children born at term (Drotar et al. 2006; Mangham et al. 2009). Preterm infants are more likely to have decreased motor skills, suffer from cerebral palsy and have poor vision and auditory function, which can impact their adult lives (Hack et al. 2002). Links have also been made between prematurity and autism-spectrum disorders (Moster, Lie & Markestad 2008).

1.1.3 Consequences of prematurity into adult life

The ongoing consequences of prematurity are difficult to determine given the relative newness of this field, however some studies have attempted to examine the effect of prematurity into adult life. A large observational trial compared 44,996

adults that were born preterm without congenital abnormalities between 1967 and 1983 in Norway to similar adults born at term (Moster, Lie & Markestad 2008). While significant advances have been made in neonatal care since that time, the trial allows us to look at the effects on adult life. Adults that were born at <28 weeks GA were significantly less likely to finish high school (68% vs 75%, relative risk (RR) 0.9, 95% confidence interval (CI): 0.8, 1.1, p=0.003) or receive a bachelor degree (25% vs 38%, RR 0.8, 95% CI: 0.6, 1.1, p=0.009) compared to those born at term. Adults born preterm were more likely to have a low income (23% vs 20%, RR 1.2, 95% CI: 0.9–1.5, p<0.001.) and receive social security benefits (20% vs 18%, RR 1.2, 95% CI: 0.9–1.5, p<0.001). They were also less likely to be married or cohabitating (10% vs 18%, RR 0.7, 95% CI 0.5–1.0, p<0.001) or to be biological parents (29% vs 43% RR 0.8, 95% CI: 0.6–1.0, p<0.001). Very low birth weight preterm infants (born weighing <1500 g) are more likely than infants born at term at 20 years of age to have a lower IQ score (87 vs 92, p<0.01) and an IQ that falls outside of the normal range; i.e. 49% of preterm infants will have abnormal mental delay (IQ <85) compared with 33% of infants born at term (p<0.01) (Hack et al. 2002). Analysis of a Swedish cohort of 635 933 adults born between 1973-1979, including 28 799 preterm births, has shown associations between prematurity and adult; asthma (Crump et al. 2011a), diabetes (Crump et al. 2011b), hypertension (Crump et al. 2011c) and prescription of psychiatric medication (Crump et al. 2010).

1.1.4 Predictors of morbidity in adult life

Preterm infants struggle to achieve adequate growth in hospital. For lack of a gold standard model, the most desirable growth trajectory for the preterm infant is to

replicate the fetus at the same gestational age (Thureen & Heird 2005). However this rarely occurs as growth often falters after birth (De Curtis & Rigo 2004; Ehrenkranz, Richard A. et al. 1999). While only a small proportion of preterm infants are born small-for-gestational age (SGA), many more are discharged home as SGA (Clark, Thomas & Peabody 2003). Infants lose roughly 10% of their birth weight in the first days of life, mainly due to a loss of extracellular fluid. Term infants balance this loss by an increase in intracellular water, which does not occur in preterm infants (Heimler et al. 1993). A recent trial of 138 preterm infants born <33 weeks GA by Collins and colleagues (2008) reported 8% and 13% were SGA for weight and length, respectively at birth. At discharge, 32% and 55% of infants were SGA for weight and length, respectively. The poorest growth was in a subgroup of infants <28 weeks GA (n=38). While only 3% were SGA for length at birth, 77% were SGA for length at discharge. There was a similar trend in SGA for weight measurements with 6% of infants SGA for weight at birth, increasing to 34% at discharge. These findings suggest that current nutritional management practices are failing to meet the growth requirements of the preterm infants. Birth weight is usually regained in the first two weeks of life but can take longer, with the most immature and sickest infants taking the longest to regain birth weight. It is this early weight loss that causes the infant to deviate from the appropriate growth trajectory. Even if the infant can maintain an adequate growth rate once birth weight is regained, the insult the infant has accrued in the immediate postnatal period can prevent an appropriate post-discharge for gestational age weight.

1.1.5 Poor neurodevelopment is associated with poor in-hospital growth

The immediate and long term consequences of poor neonatal growth in preterm infants are well documented in the literature – with poor health outcomes (Hay 2008). Infants that grow poorly have an extended hospital stay, are more prone to infection and are more likely to be re-admitted. Various co-morbidities of prematurity, such as infection and chronic pulmonary disease, are associated with both poor growth and poor neurodevelopmental outcomes. Infants who experience clinical infections, during the in-hospital period are more likely than uninfected infants to show neurodevelopmental outcomes, such as MDI <70 at 18-22 months CA (37% vs 22%, p≤0.001) and be in the 10th centile at discharge for weight (71% vs 60, p≤0.001) and head circumference (41% vs 25%, p≤0.001) (Brown et al. 2014). Thus, it is difficult to determine the contribution of growth per se to later neurodevelopmental outcomes. However, many studies have demonstrated the links between poor in hospital growth and reduced cognitive function.

Ehrenkranz and colleagues (2006) studied the association between neurodevelopmental outcome in preterm infants born between 501 and 1000 g and in-hospital weight gain. Growth velocity was calculated for the period between birth weight regained and whichever occurred first; discharge, transfer, the infant was 120 days of age or the infant weighed 2000 g. Infants in slowest weight gain quartile (12.0 g/kg/day) were more likely to have neurological impairment when compared to infants in the greatest rate of weight gain quartile (21.2 g/kg/day) at 18–22 months corrected age (CA). The infants in the slowest weight gain quartile were also more likely to recorded Mental and Psychomotor Development Index

scores <70, indicating decreased cognitive function. The same outcomes were reported when infants were separated into quartiles according to head circumference growth suggesting that weight and head circumference growth are equally suitable measurements in predicting neurological outcomes for premature infants. Similar outcomes are reported by Latal-Hajnal and colleagues (2003). Two hundred and nineteen very low birth weight (VLBW) infants (<1250 g) were assessed for growth and neurodevelopment at two years of age. Infants born in the normal weight range that experienced growth failure and were subsequently in the 10th centile for weight at two years of age showed the lowest Mental and Psychomotor Developmental Index scores. The second worst performing group were infants that were born in and remained in the 10th centile at two years of age. These findings are supported by Belfort and colleagues (2011) who examined growth outcomes in conjunction with neurological function. They found that weight, body mass index and head circumference gain in the period before term was most influential on Bayley Scales of Infant Development (2nd edition) Mental and Psychomotor developmental scores at 18 months corrected age. Growth from term to four months corrected age was associated with some improved Psychomotor but not Mental Development Index scores. There was no association between Bayley scores and growth in the 4–12 months corrected age period.

The findings of these studies highlight the importance of growth during the in-hospital period. Quality nutrition in this critical window is strongly associated with positive neurodevelopmental outcomes. Considering the lifelong consequences of poor neonatal growth, every effort must be taken to ensure growth does not falter in this critical early period.

1.2 Nutrition of the preterm infant

1.2.1 The immaturity of the preterm infant makes nutritional management difficult

The preterm infant sits in limbo, by definition no longer a fetus but neither a term infant. As such, managing their needs is often difficult. The more immature a preterm infant, the more health problems are expected with the respiratory and digestive systems often requiring the most intervention in adjusting to extra-uterine life. The fetus only begins to accumulate fat in the third trimester of pregnancy so preterm infants cannot efficiently thermoregulate and must be placed in incubators until they can maintain their temperature.

While in utero, the fetus receives a constant supply of nutrients from the mother via the placenta. These nutrients are in their simplest form and enter the blood supply to be take-up by the tissues and organs that require them. The environment is kept at a constant temperature and the fetus does not need to manage waste removal. The only dominant focus is growth. Following birth, the infant must breathe, use their digestive system, thermoregulate and adjust to the surrounding 'noise'. The difficulty of this period is greatly increased in the preterm infant.

It is difficult to determine the exact nutrient supply the fetus receives from the mother via the placenta, but well researched estimates are available (Cetin et al. 1996) (Boirie et al. 1997). Amino acids are actively transported across the placenta to the fetus at a greater rate than required for growth (Thureen & Heird 2005). Studies in fetal sheep suggest that the protein synthetic rate is two to fourfold greater than the fractional rate of growth (Meier et al. 1981). The placenta is also able to manufacture a number of amino acids for transfer to the fetus (Jansson 2001). Excess amino acids are used as an energy source (Thureen &

Heird 2005). Glucose moves freely across the placenta reflecting the rate of energy utilisation (Thureen & Heird 2005). Dependence on lipid for energy is low throughout gestation with lipid uptake only beginning in the third trimester (Thureen & Heird 2005).

Thureen and Heird state that *'replicating the body composition of the fetus of the same postconceptional age as the preterm infant undoubtedly is a more desirable nutritional goal than simply achieving the fetal rate of weight gain'* (2005, p. 96R). This statement is important because it places consequence on the quality of weight gain and growth. However, daily weight gain of the preterm infant lags well behind the estimated weight gain of the comparable fetus (Clark et al. 2003). It is estimated that intrauterine growth requires 3 g/kg/day protein, 90 kcal/kg/day carbohydrate (Thureen & Heird 2005).

Replicating fetal growth may not be an achievable aim and realistic adjustment may be needed. Determining the diet to meet the growth needs of the preterm infant can be difficult but it is likely that a combination of parenteral nutrition (if required) and fortified breast milk is optimal, especially in very preterm infants.

1.2.2 Protein must be administered early and safely to encourage growth and weight gain

Parenteral amino acids must be introduced within hours of birth to reduce the weight loss very preterm infants commonly experience in the first weeks of life. A 1000 g infant born at 26 weeks GA has approximately 88 g total protein. A diet solely composed of glucose will result in the daily loss of 1.6 g protein meaning that after seven days the infant will have lost approximately 11.2 g protein, over

10% of the in utero accumulated stores. If the infant had remained in utero, protein gain would be 1.8 g per day – 12.6 g over seven days. Therefore a week old preterm infant fed only glucose suffers a huge protein deficit compared to the comparatively aged fetus (Dusick et al. 2003).

Several studies have confirmed that early introduction of amino acids is safe and most likely beneficial to the preterm infant. Wilson and colleagues (1997) were the first to assess a more aggressive nutritional management approach. This included commencing parenteral amino acids earlier, administering amino acids at 0.5 g/kg/day at 12 hours of age advancing to 3.5 g/kg/d compared to administering 0.5 g/kg/d at day 3 increasing to 2.5 g/kg/d in 125 VLBW infants. They found that infants receiving the aggressive nutritional diet lost less weight, took less time to regain birth weight and were less likely to be discharged SGA. In a randomised controlled trial (RCT) Thureen and colleagues (2003) supplemented 28 preterm infants with 1 or 3 g/kg/day of amino acid commencing at 22 hours of life. The higher protein group showed a well tolerated, increased protein accretion. There was no difference in the amount of sodium bicarbonate given to the infants, acidosis or blood urea nitrogen concentration.

Some studies have raised concerns about early and high levels of parenteral protein for extremely low birth weight infants (<1000 g). Blanco and colleagues (2008) withdrew six infants from their trial after high blood urea levels suggested intolerance to the intervention (High: 2 g/kg/d soon after birth, daily increase 1 g/kg/d to final 4 g/kg/d n=30, Standard: 05 g/kg/d 24-36 hours post birth, daily increase 0.5 g/kg/day to 3.0 g/kg/d n=31). A follow up trial showed the high protein infants were smaller and displayed lower cognitive scores at 18 months CA (Blanco, C et al. 2008). Similarly, Clark and colleagues (2007) showed increased

blood amino acid levels with early and high level protein administration without improvements in growth. The authors discuss the benefits of avoiding negative protein balance but also remind us of the intolerance immature infants can have to additional protein.

1.2.3 High protein enteral feeding is the goal

While parenteral nutrition is often the only means of providing nutrition in the first days of life for the VLBW preterm infant, it is not a desirable long term option. The goal is to progress the infant to enteral feeds as soon as possible. Early enteral feeding is associated with decreased hospital stay along with a number of other positive benefits such as reduction in sepsis associated with intravenous therapies and allowing the parents to be more involved in caring for the infant in hospital. Because of the inability of the VLBW infant gut to absorb nutrients, parenteral nutrition must usually be provided, soon after birth, to meet the nutritional requirements. However, minimal enteral feeding is encouraged as it primes the gut to receive full enteral feeds. This approach reduces the days needed to reach full enteral feeds and length of hospital stay (Hay 2008). Although small, a recent RCT by Brumberg and colleagues (2010) showed the importance of added protein in the early neonatal period. Infants greater than 14 days of age were eligible if birth weight was ≤1250 g and they were receiving greater than 75% of their nutrition enterally (either fortified expressed breast milk (EBM), formula or combination) and had failed to regain birth weight or had a growth rate less than or equal to 15 g/kg/day. Infants received standard fortification (protein 3.2 g/kg/daty, energy 120 kcal/kg/day) and/or preterm formula (protein 3.6 g/kg/day, energy 120 kcal/kg/day). Of the 23 infants enrolled in the trial, 11 were randomised to receive

an additional enteral supplement which included both protein (protein 0.4 – additional g/kg/day) and energy (+energy 15 kcal/kg/day), while the remaining 12 received an additional enteral supplement of energy only (energy – additional 23 kcal/kg/day) in the form of medium chain triglycerides. The infants receiving the additional protein and energy supplement showed an increased growth rate (17 g/kg/day versus 11 g/kg/day) when compared to the additional energy alone group. This trial supports the need for protein for growth especially in situations when growth is faltering.

1.2.4 Human milk is recommended for preterm infants

Human milk is the optimal diet for term born infants (Callen & Pinelli 2005; Kramer et al. 2001; Tsang et al. 2005). Preterm infants fed human milk show improved immune function and neurodevelopmental outcomes (Tsang et al. 2005). Additionally, infants are able to digest and absorb nutrients more efficiently and show better gastrointestinal function (Tsang et al. 2005).

1.2.5 Major clinical concerns with enteral nutrition – necrotising enterocolitis

The immature gastrointestinal system poses a number of problems for the neonatal team. Necrotising enterocolitis (NEC) is a condition which affects the lining of the large intestine, resulting in significant levels of morbidity and mortality. Damage to the intestinal tract varies from mucosal injury to full-thickness necrosis and perforation requiring surgery. Although the rate of NEC development varies across neonatal units, usually 8–10% of infants under 1500 g or <28 weeks GA

will develop NEC. Roughly half of these infants will require emergency surgery with 50% of the infants that require emergency surgery likely to die (Kempley, Sinha & Thomas 2005).

Smaller and earlier gestation preterm infants are most at risk of developing NEC with onset occurring at any time in the neonatal unit. Risk factors include hypoxic events, ischemia, infection, bacterial colonisation and rapid advancement of feeds (Lin & Stoll 2006). While nearly all infants that go on to develop NEC have been fed enterally, the majority of enterally fed infants do not develop NEC. Delaying enteral feeds or exclusive parenteral feeds does not prevent NEC. The use of breast milk is associated with a reduction in the risk of developing NEC (Lee & Polin 2003; Lin & Stoll 2006).

1.2.6 Nutritional content of breast milk

Breast milk of women that give birth prematurely is higher in nitrogen, immune proteins, total lipid, medium chain fatty acids, energy, vitamins, some minerals and other trace elements compared to breast milk of women that deliver at term (Anderson, Atkinson & Bryan 1981; Aquilio et al. 1996; Bitman et al. 1983; Gross et al. 1980). Although the physiological reason for this difference is unknown, it has been speculated that incomplete maturation of the mammary gland allows paracellular leakage (Bitman et al. 1983). It is also possible that a variable hormonal profile may contribute.

As with term breast milk, the nutritional content of preterm breast milk declines over time (Tsang et al. 2005). While the initial days of preterm breast milk may be

adequate to meet the needs of the infant, as the nutritional content decreases the infant may experience nutritional deficit with poor growth.

In order to meet the nutritional needs of the preterm infant while utilising the known advantages of breast milk, fortifier is added to breast milk. Fortifying human milk is associated with better rates of weight gain and protein accretion and consequently the process is practised in neonatal nurseries world-wide (Kuschel & Harding 2004).

1.2.7 Breast milk fortification for preterm infant growth

Fortification of breast milk is standard practice in the developed world due to the overwhelming growth benefits to the preterm infant. The current Cochrane review (Kuschel & Harding 2004) suggests there is no reason to further compare fortified human milk to non-fortified human milk. Thirteen studies were eligible for examination in the Cochrane review. Using results from over 600 infants, the authors associate multi-nutrient fortification with short term growth improvements and gains in body weight (weighted mean difference (WMD) 2.3 g/kg/day, 95% CI: 1.7–2.9 g/kg/day), length (WMD 0.12 cm/week, 95% CI: 0.07–0.18) and head circumference (WMD 0.14 cm/week, 95% CI: 0.09–0.20 cm/week). There is a lack of evidence of long term benefits of multi-nutrient fortification of breast milk and this is where the authors suggest research should be directed, along with altered components of the fortifier to achieve the optimal composition for quality growth.

1.2.8 Quality of protein in human milk fortifier

The quality of the protein supplement used in HMF, particularly the whey:casein

ratio, is important in preterm infants because this affects protein digestion, absorption and the pattern of plasma amino acids that results. Protein supplements in HMF are typically hydrolysed are there is evidence that this aids digestion (Mihatsch, Hogel & Pohlandt 2001). Unlike human milk protein which has a whey:casein ratio of ~70:30, bovine milk protein has a ratio of 20:80. Casein protein contains higher amounts of phenylalanine and tyrosine and is more easily digested. Whey protein produces higher but shorter peaks of amino acids, which may aid protein synthesis, while casein protein produces a slower increase in amino acids which tends to more prevent protein breakdown, at least in adults (Boirie et al. 1997; Brown et al. 2014)

1.2.9 RCT of protein in human milk fortifier (HMF)

A small number of randomised controlled trials have compared growth outcomes of preterm infants when infants have been randomised to different concentrations of protein fortification. The aim of this section is to review the quality of studies in preterm infants in which enteral protein content was varied and growth was assessed as the primary outcome.

A comprehensive literature search was undertaken using Medline (PUBMED), CINAHL, Embase and CENTRAL. Trial quality was assessed using criteria adapted from the Cochrane risk of bias assessment (Higgins et al. 2011) and the SIGN checklist for randomised controlled trials (Harbour & Miller 2001).

Trials were included if participants were born preterm (<37 weeks GA), were randomised to receive different diets, a nutritional intervention involving an

increased amount of protein was administered enterally and growth was a primary outcome.

This literature search was conducted from January–June 2012 using a combination of medical subject heading (protein dietary, dietary supplement, nutritional requirement, breast milk expression, milk human, weight gain, infant premature) and text words (human milk fortifier). Four hundred and seven journal articles were found. Each abstract was read carefully to assess eligibility and 15 journal articles were read in full. Ten were rejected due to not meeting the inclusion criteria (Table 1.1) therefore, five were eligible to be included in this review (Table 1.2).

The authors of the included studies used birth weight and/or gestational age for eligibility criteria. All infants were born <34 weeks GA. Not all included studies had birth weight eligibility criteria. Reis et al. (2000) and Porcelli et al. (2000) included infants that were born <1500 g and <1600 g, respectively.

Table 1.1 Table of excluded studies

	Reason for exclusion
Rochow et al. (2011)	Primary outcome incidence of metabolic acidosis
Martins and Krebs (2009)	Compared protein to unfortified EBM
Mukhopadhyay, Narang and Mahajan (2007)	Compared protein to unfortified EBM
Embleton, N.D. and Cooke, R.J. (2005)	Formula
Polberger, S et al. (1999)	No difference in protein values
Wauben, Gibson and Atkinson (1999)	Primary outcome zinc retention
Lucas et al. (1996)	Formula
Sankaran et al. (1996)	No difference in protein values
Metcalf et al. (1994)	No difference in protein values
Goldman et al. (1969)	Formula

1.2.9.1 Nutritional interventions of studies summarised in Table 1.2

Reis and colleagues (2000) compared 0.9 g protein/100 ml EBM supplementation with 0.6 g protein/100 ml EBM. Porcelli and colleagues (2000) compared 1g protein/100 ml EBM supplementation with a group receiving 0.7 g protein/100 ml EBM. Miller and colleagues (2012) randomised infants to receive either intervention fortifier with 1.4 g protein/100 ml EBM compared with standard fortifier, adding 1.0 g protein/100 ml EBM. Tan and colleagues (2008) were the only group not to publish the raw amount of protein added, only publishing intended intake values including EBM. Infants received either high protein feed of 4 g/kg/day, or control feed of 3.3 g/kg/day. Arslanoglu and colleagues (2006) were

the only group to individualise protein fortification. Infants were randomised to an adjusted protein group (n=17) or a standard protein group (n=17). Protein intake for the adjusted group was calculated based on twice-weekly blood urea nitrogen (BUN) levels. At baseline 5 g HMF/100 ml EBM was administered giving 0.8 g protein/100 ml EBM. At level one, 6.25 g HMF/100 ml EBM was given. At levels two and three, an extra 0.4 and 0.8 g protein/100 ml EBM, respectively was given. Every time the BUN levels were <3.2 mmol/L protein fortification was increased by one level. If the BUN level was >5.0 mmol/l protein fortification was decreased by one level. In the third week of the trial total protein intake – protein from fortifier and EBM combined – in the adjustable group was 2.3 g protein/100 ml EBM compared to 1.9 g protein/100 ml EBM.

Miller et al. (2012) and Reis et al. (2000) both matched the caloric intake of the two trial arms to ensure the groups were isocaloric. Porcelli et al. (2000) and Tan and Cooke (2008) provided a control product which was higher in calories than the intervention product.

Miller (2012) and Arslanoglu (2006) measured EBM samples during their studies, allowing an accurate protein intake to be reported. Both calculated protein by measuring nitrogen using the Kjeldahl method (Protein g/l = nitrogen x 6.38, assuming 27% of non-protein nitrogen was bioavailable (Fomon 1991)). Tan and Cooke (2008) assumed a protein content for breast milk but did not state the value in the paper. Reis (2000) assumed a protein content of 1.4 g/100 ml EBM. Porcelli (2000) assumed a protein content of 1.76 g/100 ml EBM.

Table 1.2 Randomised controlled trials of preterm infants with high protein fortification intervention and growth as a key outcome

Reference and inclusion criteria	Randomisation point, Baseline characteristics	Intervention	Outcome	Trial quality
(Miller et al. 2012) Australia <31 weeks GA	Randomised before receiving or within 3 days of receiving standard fortifier Intervention: mean GA 27.5 weeks mean birth weight 1012 g age at randomisation 12 days Control: mean GA 28 weeks mean birth weight 1056 g age at randomisation 13 days	Intervention: High protein fortifier added when intake 80 ml/kg/day High protein fortifier 1.4 g protein/100 ml n=43 Median intake over first 28 days 4.2 g/k/d Control: 1 g protein /100 ml Median intake over first 28 days 3.6 g/k/d n=49 Isocaloric Hydrolised whey protein	Primary outcome: Length gain (cm/week) on discharge home/EDD No differences in length, weight or head circumference gain Less infants were discharged SGA for length in the intervention group	Sequence generation: A Allocation concealment: A Treatment Blinding: A Assessment blinding: A Complete data outcome: Y
(Arslanoglu, Moro & Ziegler 2006) Italy	Randomised before reaching intake 90 ml/kg/day. Intervention:	Intervention: If twice weekly BUN measurements ranged between 9 and 14 mg/dl no adjustments were made. If BUN was <3.2 mmol/L,	Primary outcome: Weight gain from trial start (feeding at 150 ml/kg/day) to 2000 g Intervention infants gained	Sequence generation: U Allocation concealment: A

<34 weeks GA 600–1750g	mean GA 31.8 weeks mean birth weight 1386 g age at randomisation 18 days Control: mean GA 31.3 weeks mean birth weight 1407 g age at randomisation 18 days	fortification was increased one level. If BUN was >5mmol/L fortification was decreased by one level. Level: Amount added g/100 ml EBM L3: HMF 6.25+ protein 0.8 L2: HMF 6.25+ protein 0.4 L1: HMF 6.25 L0: HMF 5 L-1: HMF 3.75 L-2: HMF 2.5 Intervention mean protein intake Week 1 1.9 g/100 ml EBM 2.9 g/kg/day Week 2 2.2 g/100 ml EBM 3.2 g/kg/day Week 3 2.3 g/100 ml EBM 3.4 g/kg/day n=17	more weight per day and per kg per day. Interventions infants showed greater head circumference gain (mm/day)	Treatment Blinding: N Assessment blinding: N Complete data outcome: N

		Control mean protein intake Week 1 1.9 g/100 ml EBM 2.9 g/kg/day Week 2 2.0 g/100 ml EBM 2.9 g/kg/day Week 3 1.9 g/100 ml EBM 2.8 g/kg/day n=17 Additional protein calories in adjustable protein group were not matched in standard group bovine whey concentrate		
(Tan & Cooke 2008) UK <29 wks GA	Randomised within 7 days of age Intervention: mean GA 26.0 weeks mean birth weight 911 g age at randomisation U Control: mean GA 26.2 weeks mean birth weight 914 g age at randomisation U	Intervention: High protein parenteral nutritional beginning within 24 hours of birth and enteral nutrition when milk intake >75 ml/kg/day. Parenteral intake was ceased when infant receiving >50% total fluid as milk High protein fortifier 4 g/kg/day, 133–150 kcal/kg/day n=55	Primary outcome: Head circumference gain from birth to 36 weeks post menstrual age No growth differences were observed.	Sequence generation: A Allocation concealment: A Treatment Blinding :N Assessment blinding: N Complete data outcome: N

		Control: Fortifier protein 3.3 g/kg/day, 133 kcal/kg/day n=59 Additional protein calories in adjustable protein group were not matched in standard group. Nutricia (Cow and Gate)		
(Reis et al. 2000) USA ≤33 wks GA, ≤1600 g.	Randomised within 21 days of age. Intervention: mean GA 29.4 weeks mean birth weight 1247 g age at randomisation U Control: mean GA 29.7 weeks mean birth weight 1274 g age at randomisation U	Intervention: High protein fortification began when intake ≥100 ml/kg/day Intervention: 0.9 g/100 ml EBM, n=64 Control: 0.6 g/100 ml EBM, n=55 Isocaloric Similac HMF, Ross Laboratories	Primary outcome: Weight gain from trial day 1 to trial day 29/hospital discharge. High protein infants showed greater weight gain and length gain, and weighed more at trial end. In a sub-group, per protocol analysis, infants in the higher protein group showed increased weight gain length gain and head circumference gain	Sequence generation: U Allocation concealment: U Treatment Blinding: A Assessment blinding: A Complete data outcome: N
(Porcelli et al. 2000) USA	Infants were randomised on reaching intake 150 ml/kg/day Intervention:	Intervention: High protein fortification began when fortifier would be introduced under standard practice	Primary outcome: Weight gain from intake 150 ml/kg/day to weaned to unsupplemented milk	Sequence generation: U Allocation concealment: U

25–32 wks GA, 600–1500 g.	mean GA 29.3 weeks mean birth weight 1257 g age at randomisation 15.3 days Control: mean GA 29.0 weeks mean birth weight 1193 g age at randomisation 14.1 days	Intervention: 1 g protein /100 ml EBM n=47 Control: 0.7 g protein/100 ml EBM n=43 Control HMF contained more calories than Intervention. Bovine whey, Wyeth Nutritionals	Intervention infants showed increased weight gain (g/day, g/kg/day) and increased head circumference gain (cm/week)	Treatment Blinding: N Assessment blinding: A Complete data outcome: N

A: adequate, Y: yes, N: no, U: unknown, GA: gestational age, EDD: estimated delivery date, EBM: expressed breast milk

1.2.9.2 Outcomes of studies summarised in Table 1.2

Arslanoglu and colleagues (2006) showed increased weight (30.1 ± 5.8 vs 24.8 ± 4.8 g/d, 17.5 ± 3.2 vs 14.4 ± 2.7 g/kg/d) and head circumference gain (1.4 ± 0.3 vs 1.0 ± 0.3 mm/d) in the intervention compared to the control group. Porcelli and colleagues (2000) also found infants in the high protein group showed greater weight gain (26.8 ± 1.3 vs 20.4 ± 1.2 g/d, 19.7 ± 0.98 vs 16.8 ± 0.96 g/k/d) and head circumference gain (1.0 ± 0.1 vs 0.8 ± 0.1 cm/wk). Reis and colleagues (2000) showed greater weight gain per kilogram per day (17.6 ± 4.1 vs 14.9 ± 3.2 g/kg/day). Miller et al. (2012) and Tan and Cooke (2008) did not show increased growth gains. Interestingly, Miller (2012) showed less infants discharged SGA for length in the high protein group when compared to control. At birth, 16% (7/43) of high protein infants were SGA for length compared with 14% (7/49) of standard protein infants. At discharge 49% (21/43) of high protein infants were SGA for length compared with 63% (31/49) (p=0.047). Less infants in the high protein group being classified SGA for length at discharge suggests they were growing better despite no differences in length gain/week and overall length.

1.2.9.3 Quality of studies summarised in Table 1.2

Three studies adequately blinded caregivers and researchers to the intervention (Miller et al. 2012; Reis et al. 2000; Tan & Cooke 2008). Two were open due to the nature of administering the intervention (Arslanoglu, Moro & Ziegler 2006; Porcelli et al. 2000).Miller et al. (2012) and Tan and Cooke (2008) were the only studies to include adequate sequence generation and allocation concealment methodologies. Complete data outcome was poorly managed by Porcelli and colleagues (2000). Despite ninety infants being enrolled and randomised, once the

trial sample size of 64 was reached the trial ceased with data reported for the 64 infants only. Reis and colleagues (2000) randomised 144 infants, of which 119 reached 'trial day one'. Explanation was provided for 15 of the infants not reaching trial day one – the infant did not receive an HMF feed and intolerance (7 infants). However no explanation is provided for the remaining infants not reaching trial day one. Data on the 119 infants is presented incorrectly as intention to treat. Tan and colleagues (2008) randomised 142 infants but only analysed data from 104 infants, excluding infants that died before trial end (36 weeks post menstrual age). Arslanoglu and colleagues (2006) randomised 36 infants, but analysed 34, with two infants leaving the trial due to feeding intolerance in terms of abdominal distension and emesis.

Porcelli and colleagues (2000) were supported by the manufacturers of the high protein fortifier used in the trial, but did not divulge any detailed information on the manufacturers' involvement in trial design, management, data analysis and manuscript preparation. Miller and colleagues (2012) received donated trial products from Nestle Nutrition.

1.2.9.4 Summary of findings of studies in Table 1.2

These fortification studies show that increased protein is normally beneficial for growth. What remains to be determined is the optimal protein content HMF required to replicate in utero growth.

A thorough analysis highlights the importance of adhering to the CONSORT guidelines when planning a trial. It is important that researchers, care givers, participants and statisticians are blinded to treatment group allocation and

sequence generation. The trial solutions must also be isocaloric to ensure that the protein is the only difference between the treatment groups. Adequate data follow up is important to maintaining the integrity of the randomisation, such that if an infant is randomised the data for that infant must be analysed regardless of participation in the trial. If support is received from an industry company that is not involved in trial design or analysis it is important that this is clearly reported in any resulting publications.

None of the studies summarised provided HMF with a protein content that meets the amount currently recommended for optimal growth. The European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) guidelines state that infants born less than 1000 g require 4.0–4.5 g/kg/day of protein and infants born 1000–1800 g require 3.5–4.0 g/kg/day of protein (Agostoni et al. 2010). The guidelines emphasise that excess protein intake will not be detrimental to the infant but that protein deficit will impair growth. Miller et al. (2012) administered an intervention that reached a median protein intake of 4.2 g/kg/day but had inconclusive growth results, suggesting that a trial with increased protein intake is required.

1.2.10 Good quality nutrition is vital to achieving good quality growth

The literature suggests that high amounts of protein, whether given parenterally or enterally, in formula or as fortified breast milk, is both tolerated by the infant and necessary for good quality infant growth. Recent attention has also focused on the quality of the protein, ensuring richness in essential specific amino acids. There is

still work to be done in this field but it is an important issue for the researcher and clinician to be aware of (Johnson et al. 2012).

1.2.11 The challenge of fortifying direct breast feeds

The preterm infant cannot coordinate the sucking-swallowing motion required for direct breast feeds until around 32–34 weeks post menstrual age. Establishing and encouraging direct breast feeds is important for both the infant and the mother. The naso-gastric tube is vital to establishing enteral nutrition in the early period of infant life but it is not a sustainable or ordinary way for the infant to feed after hospital discharge. Mothers often find it difficult to bond with their offspring in the traumatic intensive care environments where preterm infants spend weeks or months (Wigert et al. 2006). Direct breast feeds allow mothers to connect to their child, feel involved in their care and establish a sense of normalcy (Boucher et al. 2011). Current standard practice does not allow direct breast feeds to be fortified, meaning that the infant misses out on essential nutrition for that feed.

While introduction and continuation of direct breast feeding is vital to infant development, administering appropriate nutrition is also of high importance. To reconcile these two practicalities, a possible solution is to provide the extra nutrients mixed with water and given separately to the feed, however no published reports of this practice in the preterm infant were available at the time of preparation of this thesis.

1.3 Body composition

1.3.1 Importance of determining body composition

Poor quality growth in the fetal and neonatal period is associated with poor adult health. Preterm infants are often discharged with more fat than term infants (Roggero et al. 2009). A recent systematic review suggests that this may be due to a decrease in the accumulation of fat-free mass rather than an increase in fat mass accumulation (Johnson et al. 2012). Weight gain that is mostly fat increases the risk of metabolic disease in adult life (Singhal et al. 2002; Thureen 2007). Conversely, many preterm infants fail to thrive after birth and fall below the 10th centile even if birth weight was appropriate for gestational age. As discussed in section 1.1.5, poor growth is associated with poor cognitive development. Given the importance of the quality of growth, body composition models give great insight into the effect of macronutrient intake on body composition. The quality of infant growth and encouraging lean tissue growth with appropriate fat depositions should be the nutritional goal of the neonatal unit.

1.3.2 Body composition measurement methods

There are a variety of body composition methods available to clinicians and researchers providing various levels of accuracy and precision with varying degrees of impact on the infant (Table 1.3).

1.3.2.1 Imaging techniques

Computed tomography (CT), magnetic resonance imaging (MRI) and dual energy X-ray absorptiometry (DXA) are imaging methods that provide a three compartment model of body composition; fat mass, fat-free mass and bone (Table 1.3). CT provides a detailed analysis of the three compartment model, differentiating between adipose, muscle, skin, viscera and bone and allowing the location of adipose tissue to be identified. Whilst both accurate and precise, CT subjects the preterm infant to potentially unsafe levels of radiation and is only used in necessary diagnostic procedures. MRI provides an image similar to that of CT without the radiation. For a quality MRI to be produced the infant needs to remain still for roughly 30 minutes. While sedation is an option, an MRI is normally only performed for clinical diagnostic procedures not observational measures. DXA uses the typical X-ray principle of placing an X-ray source on one side of an object and measuring the intensity on the other which is altered by thickness, density and chemical composition. Preterm infants typically experience multiple X-ray procedures monitoring the placement of catheters and endotracheal tubes. A typical preterm infant receives 11 X-rays with the median cumulative dose of radiation 138 μSv in the neonatal unit (Donadieu et al. 2006). While the single radiation dose from DXA is low (<3 μSv) (Roggero et al. 2007), accumulated radiation means that it may not be suitable for repeated measurements. While these methods provide high quality images they are unsuitable for the preterm infant. All are expensive, require transportation of the infant to the machine and cannot be used on infants that are not self-ventilating, as respiratory support apparatus cannot be used in conjunction with these methods.

1.3.2.2 Techniques using a two compartment model

There are a variety of two-component techniques which determine fat mass and fat-free mass (Table 1.3). One of the most simplistic is the Underwater weigh, based on the Archimedes Principle which entails submersion in water and recording the amount of water displaced. This measurement can be combined with a laboratory weight and used to calculate the density and fat mass of the body. However, the patient needs to hold their breath, making this method clearly unsuitable for preterm infants and children. Air-displacement plethysmography (ADP) uses similar principles. The device consists of two chambers, the patient sits in one and a second serves as a reference volume (Ellis, Kenneth J. 2007). A diaphragm separates the two chambers and oscillates to alter the volume of each chamber. While originally designed for adults, a smaller ADP machine, the Pea-Pod, has been used to measure body composition of term and stable preterm infants. Unstable infants are unable to safely use this method as the infant must be transported to the Pea-Pod, precluding infants that require ventilator support and minimal handling (Ellis, K. J. 2000). Also, due to the need for an air tight seal on the Pea-Pod, monitoring leads such as heart rate monitors and ventilation tubes are not permissible. Though the Pea-Pod is an accurate measure of body composition it fails the preterm population by excluding unstable infants that are most commonly the infants experiencing poor growth (Ellis, K. J. 2000).

A commonly used indirect method of measuring body composition is the dilution method which is the gold standard for determining total body water, allowing the volume of fat-free mass to be obtained (Ellis, K. J. 2000). The principle behind the dilution method is that the volume of a compartment can be defined as the ratio of the dose of a stable isotope tracer to its concentration in that body compartment. A

blood sample is collected before administration of the tracer to detect any background levels. A second blood sample is collected after the tracer dose has equilibrated, which takes approximately three hours. The amount of tracer in the blood samples is then measured using spectroscopy and total body water (and thus fat-free mass) can then be determined. While the dilution method has advantages over other body composition methods there are some aspects that make it disagreeable for preterm infants. On top of daily routine blood test two additional blood tests are required which can be distressing for both the infant and parents. The results are not instant and must be sent to a laboratory, adding to the time and cost required. Restrictions are also placed on how often the test can be performed as the tracer remains in the body for 10–14 days.

A further method for determining body composition is Bioelectrical Impedance Spectroscopy (BIS) (Ellis, K. J. 2000). BIS is a new multi frequency measuring technique that assesses body water volumes based on the measurement of their opposition (impedance) to the flow of an electrical current. A detailed description of this method follows this Literature Review in Chapter 2.

BIS is an advanced measuring technique, based on the principles of the single frequency Bioelectrical Impedance Analysis (BIA). The BIS technique is potentially advantageous in the preterm infant population in that it is non-invasive and results are instant. The technique can be performed while the infant remains in the cot, connected to all necessary monitoring, intravenous and ventilatory support lines. While used routinely in adults and children, BIS has yet to be validated in preterm infants (Ellis, K. J. 2000).

Table 1.3 Advantages and disadvantages of current body composition assessment methods

	Exposure to radiation	Measure distribution of fat	Portable	Simple analysis	Instant results	Suitable for unstable infants	Validated in preterm infants
Computed tomography (CT)	✓	✓	X	X	✓	X	✓
Magnetic resonance imaging (MRI)	X	✓	X	X	✓	X	✓
Dual energy X-ray (DXA)	X	✓	X	X	✓	X	✓
Underwater weigh	X	X	X	X	✓	X	✓
ADP	X	X	X	X	✓	X	✓
Pea-Pod	X	X	✓	X	✓	X	✓
Dilution	X	X	✓	X	X	✓	✓
BIS	X	X	✓	✓	✓	✓	X

1.3.3 Intervention studies and body composition assessment in preterm infants summarised

A number of trials have compared the effect on body composition of different nutritional interventions in preterm infants and are summarised in Table 1.4. No published review exists of preterm infant nutritional interventions where body composition has been analysed so it is important to document the studies in this literature review.

Trials were included if preterm infants were randomised to receive any nutritional intervention and recorded body composition as a key outcome. If other growth parameters were described they are also recorded in the table. There is large variation in time periods of intervention and measurement, intervention diets and participant numbers.

1.3.3.1 Included publications

Ten publications reporting nine separate studies were included in this review and are summarised in Table 1.4.

1.3.3.2 Participants summarised in Table 1.4

All the infants studied were <35 weeks GA and <2000 g. The most immature infants studied were <32 weeks GA and <1500 g.

1.3.3.3 Nutritional Interventions summarised in Table 1.4

The dietary interventions summarised in Table 1.4 vary in composition, number of groups and duration and include post discharge feeding, in-hospital protein and omega-3 fatty acids. Three trials compared an enriched formula to standard formula after discharge (Amesz et al. 2010; Cooke, Griffin & McCormick 2010; De Curtis, Pieltain & Rigo 2002). Lapillonne et al. (2004) and Embleton and Cooke (2005) both compared isocaloric formulas that contained differing amounts of protein commencing when full enteral feeds were reached. Lapillonne et al. (2004) compared a group receiving standard protein 2.0 g/100 ml of formula to a group receiving 2.2 g/100 ml to the infant's EDD. Infants in the Embleton and Cooke (2005) trial were randomised into 3 groups and received either formula containing 2.2 g/100 ml, 2.4 g/100 ml or 2.6 g/100 ml of protein to 12 weeks CA. Costa-Orvay and colleagues (2011) looked at altering both protein and energy content of in-hospital formula. Infants received 3.7 g/kg/d protein and 129 kcal/kg/d, 4.2 g/kg/d protein and 150 kcal/kg/d or 4.7 g/kg/d and 150 kcal/kg/d. Two studies examined the effects of Docosahexaenoic Acid (DHA). Groh-Wago and colleagues (2005)

compared infants that consumed milk or formula (mother's choice) fortified with DHA and Arachidonic acid from fish/fungal oil or egg/fish oil. Kennedy and colleagues (2010) compared long chain polyunsaturated fatty acids (LCPUFA) enriched formula to standard formula. Wauben et al. (1998) and Aimone et al. (2009) compared fortified breast milk to unfortified breast milk post hospital discharge.

1.3.3.4 Body composition assessment summarised in Table 1.4

Of the included studies, eight used DXA to determine body composition. Costa-Orvay et al. (2011) used BIA. The remaining trial (Kennedy et al. 2010) used skinfold, deuterium and bioelectrical impedance measures to determine body composition.

1.3.4 Outcome assessments summarised in Table 1.4

The time points assessed differed greatly between the groups. All time points are given as the infant's corrected age.

Six studies measured infants at term or discharge (Amesz et al. 2010; Cooke, Griffin & McCormick 2010; Embleton, N.D. & Cooke, R.J. 2005; Lapillonne et al. 2004; Wauben et al. 1998). Costa-Orvay and colleagues (2011) – the only group to measure infants in hospital – also measured the infants at 28 days of age. De Curtis and colleagues (2002) were the only trial to measure infants at two months of age. Three studies measured infants at 12 weeks of age(Cooke, Griffin & McCormick 2010; Embleton, N. D. & Cooke, R. J. 2005; Wauben et al. 1998). Aimone and colleagues (2009) conducted the only trial to measure infants at four

months of age. Two studies measured infants at six months of age (Amesz et al. 2010; Cooke, Griffin & McCormick 2010). Four studies measured infants at 12 months of age (Aimone et al. 2009; Cooke, Griffin & McCormick 2010; Groh-Wargo et al. 2005; Wauben et al. 1998). One trial measured infants at 10 years of age (Kennedy et al. 2010).

1.3.4.1 Quality of trials summarised in Table 1.4

The trials included are all randomised controlled trials but the quality is ambiguous in some cases due to limited information. Blinding was adequate in the enriched formula studies (Amesz et al. 2010; Cooke, Griffin & McCormick 2010; Costa-Orvay et al. 2011; De Curtis, Pieltain & Rigo 2002), the isocaloric protein studies (Embleton, N. D. & Cooke, R. J. 2005; Lapillonne & Salle 1999; Lapillonne et al. 2004) and DHA studies (Groh-Wargo et al. 2005; Kennedy et al. 2010). Blinding was not feasible in the fortified breast milk trials. Sequence generation and allocation concealment were not always explained in detail and for a number of trials is judged 'unclear'.

All but three of the studies (Costa-Orvay et al. 2011; De Curtis, Pieltain & Rigo 2002; Lapillonne et al. 2004) show incomplete data outcomes. The majority of trials lost participants to follow-up visits though this was distributed evenly across randomised groups.

1.3.4.2 Summary of findings summarised in Table 1.4

Generally, the overall quality of the trials was good, although more information is needed regarding randomisation. Blinding was adequate where possible and follow up rates were reasonable considering the population and length of the trials.

There is an urgent need for a randomised controlled trial involving fortification of breast milk during the in-hospital period focusing on quality of growth. The trials examined in Table 1.4 focus on the growth of the infant following hospital discharge. While this period is very important in establishing good adult health, the period from birth to hospital discharge, which has been established as a vital window for neurological development, has been severely neglected (Belfort et al. 2011). The first weeks of life are vital in establishing good quality growth by administering the best possible nutrition. Measuring this growth by a population appropriate body composition technique is vital in strengthening knowledge in this field. This summary highlights an important gap in the literature that there are no nutritional intervention studies that have used serial body composition measurements to track the accruement of favoured lean mass.

Table 1.4 Summary of randomised controlled nutritional intervention trials with body composition measurements

Reference	Setting, birth inclusion criteria	Number of subjects	Randomisation point, baseline characteristics	Intervention	Method	Time point (CA)
(Cooke, Griffin & McCormick 2010)	UK, tertiary perinatal centre ≤34 weeks GA, ≤1750 g	Total randomised 139 (+ 25 breast fed reference group) Intervention A Term – 56 12 weeks – U 6 months – U 12 months – U Intervention B Term – 57 12 weeks – U 6 months – U 12 months – U Intervention C Term – 26 12 weeks – U 6 months – U 12 months – U	Randomised immediately prior to discharge A: mean GA 31.3 weeks mean BW 1414 g age at randomisation U B: mean GA 30.9 weeks mean BW 1402 g age at randomisation U C: mean GA 30.7 weeks mean BW 1349 g age at randomisation U	A: Preterm formula 80 kcal/100 ml Protein 2.2 g/100 ml Ca 108 mg/100 ml P 54 mg/100 ml B: Term formula 66 kcal/100 ml Protein 1.4 g/100ml Ca 54 mg/100 ml P 27 mg/100 ml C: Preterm formula to term then term formula to 12 months	DXA	Term 12 weeks 6 months 12 months
(Amesz et al. 2010)	The Netherlands, neonatal unit	Total randomised 102 (+50 breast fed reference infants)	Randomised at term A: mean GA 30.7 weeks Mean BW 1345 g	A: Post discharge formula 67 kcal/100 ml protein 1.7 g carbohydrate 7 g	DXA	Term 6 months

	<32 weeks GA, <1500 g	Intervention A Term – 52 6 months – 43 Intervention B Term – 50 6 months – 34	age at randomisation 40.7 weeks B: mean GA 30.9 weeks Mean BW 1377 g Age at randomisation 40.4 weeks	fat 3.5 g B: Term formula 67 kcal/100 ml protein 1.47 g carbohydrate 7.2 g fat 3.5 g		
(De Curtis, Pieltain & Rigo 2002)	Setting unknown <35 weeks GA, <1750 g	Total randomised 33 Intervention A Discharge – 16 2 months – 16 Intervention B Discharge – 17 2 months – 17	Randomised prior to discharge A: mean GA 30 weeks mean BW 1294 g age at randomisation 45 days weeks B: mean GA 30 weeks mean BW 1261 g age at randomisation 45 days	A: Post discharge formula 74 kcal/100 ml protein 1.8 g/100 ml carbohydrate 7.5 g/100 ml fat 4.1 g/100 ml B: Term formula 66 kcal/100 ml protein 1.4 g/100 ml carbohydrate 7.1 g/100 ml fat 3.6 g/100 ml	DXA	Discharge 2 months
(Wauben et al. 1998)	Canada <1800 g, AGA	Total randomised 25 (+ control group 12) Intervention A Randomisation – 12 Term – 11 3 months – 7 6 months – 9 12 months – 7	Randomised when 80% of feed breast milk A: mean GA 29.9 weeks mean BW 1400 g age at randomisation 33 weeks B: mean GA 30.1 weeks mean BW 1300 g	A: Breast milk + multi-nutrient fortifier (12) Protein 3.7 g/1000 ml carbohydrate 34.7 g/1000 ml Ca 15.2 mmol/1000 ml P 14.1 mmol/1000 ml B: Breast milk + Ca and P alone Ca 15.2 mmol/1000 ml	DXA	Term 12 weeks 6 months 12 months

		Intervention – B Term – 13 Discharge – 13 3 months – 10 6 months – 9 12 months – 9	age at randomisation 33 weeks	P 14.1 mmol/1000 ml		
(Aimone et al. 2009)	Canada, neonatal intensive care units <33 weeks GA, <1800 g	Total randomised 39 Intervention A 4 months – 17 12 months – 16	Randomised immediately prior to discharge A: mean GA 29.8 weeks mean BW 1322 g age at randomisation U	A: Fortified breast milk	DXA	4 months 12 months
(Embleton, N. D. & Cooke, R. J. 2005)	UK, special care baby unit ≤34 weeks GA, ≤1750 g	Total randomised 77 Intervention A Discharge – 22 12 weeks – 19 Intervention B Discharge – 23 12 weeks – 18 Intervention C Discharge – 23	Randomised at 3 weeks of age A: mean GA 30 weeks mean BW 1386 g age at randomisation U B: mean GA 30 weeks mean BW 1379 g age at randomisation U C: mean GA 31 weeks	Isocaloric formulas 80 kcal/100ml A: 2.6 g/100 ml B: 2.4 g/100 ml C: 2.2 g/100 ml	DXA	Discharge 12 weeks

		12 weeks – 21	mean BW 1414 g age at randomisation U			
(Lapillonne et al. 2004)	France, neonatal intensive care unit <32 weeks GA, <1600 g	Total randomised 41 Intervention A Discharge – 19 Term – 19	Randomised within 3 weeks of age A: mean GA 29.6 weeks mean BW 1231 g age at randomisation 13 days	A: High protein formula 2.2 g/100 ml (21)	DXA	Discharge Term
(Groh-Wargo et al. 2005)	USA, neonatal intensive care unit <33 weeks GA, <1800 g	Total randomised 60 Intervention A 12 months – 12 Intervention B 12 months – 12	Randomised at within 5 days of age A: mean GA 30.6 weeks mean BW 1424 g age at randomisation 5 days B: mean GA 30.4 weeks mean BW 1363 g age at randomisation 3 days	Infant formula and/or breast milk A: DHA (fish/fungal) B: DHA (egg/fish)	DXA	12 months
(Kennedy et al. 2010)	UK, neonatal intensive care units <35 weeks GA, <2000 g	Total randomised 238 Intervention A 10 years – 50	Randomised before formula feeding commenced A: mean GA U mean BW U	A: LCPUFA supplemented formula	Skinfold BIA Deuterium	10 years

			age at randomisation U			
(Costa-Orvay et al. 2011)	Spain, neonatal intensive care units <33 weeks GA, <1500 g	Total randomised 32 Control group A – 8 Intervention B – 12 Intervention C – 12	Randomised when birth weight had been regained A: mean GA 29.6 weeks mean BW 1196 g age at randomisation U B: mean GA 30.2 weeks mean BW 1220 g age at randomisation U C: mean GA 29.5 weeks mean BW 1313 g age at randomisation U Control: mean GA 29 weeks mean BW 1138 g age at randomisation U	A: Formula protein 3.7 g/kg/day, 129 kcal/kg/day B: Formula protein 4.2 g/kg/day, 150 kcal/kg/day C: Formula protein 4.7 g/kg/day, 150 kcal/kg/day	BIA	28 days

GA: Gestational Age, AGA: Appropriate for Gestational Age, DHA: Docosahexaenoic Acid, LCPUFA: Long Chain Polyunsaturated Fatty Acids, U: unknown

1.4 Rationale for thesis

Summarising the current literature on preterm infant nutrition exposes a sizeable gap in the knowledge base. There is an urgent need for in-hospital based randomised controlled trials of high protein HMF with repeated body composition measurements. Good quality growth in this time is necessary for optimal neurodevelopmental and metabolic health in adult life. Nutrition must begin soon after birth if postnatal growth failure is to be avoided. Breast milk is optimal for infants but it must be correctly fortified to provide the nutrients the preterm infant requires. Direct breast feeds should be encouraged despite the infant not receiving fortified breast milk for that feed. The present study proposes a small volume, high protein, nutrient rich 'entrée' which will precede the infant's normal feed. This approach will allow researchers to accurately predict the amount of protein the baby is receiving and ensure that when the infant moves to partial and then full breast feeds, nutrition is adequate.

1.5 Aims of this project

The current study is addressing two aims, which are ordered by the work of larger magnitude first:

1. To conduct a randomised controlled trial comparing weight gain in breast fed preterm infants fed a high protein content human milk fortifier compared with standard protein content human milk fortifier. In order to address the issue of fortifying direct breast feeds the fortifier will be given as an entrée before the feed.

2. Develop preterm infant specific constants to allow body composition to be calculated using Bioelectrical Impedance Spectroscopy.

Developing resistivity constants and validating Bioelectrical Impedance Spectroscopy in preterm infants

Chapter 2 Developing resistivity constants and validating Bioelectrical Impedance Spectroscopy in preterm infants

The candidate was responsible for the study conduct, assisting the neonatal research nurses in recruiting and the measuring of recruited infants; data collection, management and cleaning, storage of samples, demographic statistics, bromine analysis, interpretation of results and manuscript preparation.

2.1 Introduction

Infants who gain mass rapidly in the neonatal period are more likely to experience poor adult metabolic health (Boirie et al. 1997). Because of their size and need for incubation, body composition measurement methods that are suitable for adults are not appropriate for preterm infants. Bioelectrical Impedance Spectroscopy (BIS) is potentially the most suitable for preterm infants as it is immediate, inexpensive, non-invasive and can be performed cot-side. BIS is a recently developed technique which estimates total body water (TBW), allowing fat-free and fat mass values to be calculated. The gold standard for determining fat-free mass (FFM) is the well documented deuterium dilution method (Ellis, K. J. 2000) where TBW is determined, allowing FFM to be derived. Extracellular water (ECW) can also be determined using sodium bromide as the tracer. However these methods are time-consuming, expensive and invasive.

2.2 Bioelectrical Impedance Spectroscopy principles

BIS operates on the principle that electricity flows easily through conductive media such as body water in the lean or FFM but encounters resistance (impedance) in less aqeous tissues such as adipose tissue. Resistance is the opposition to flow of an electric current and is dependent upon the inherent conductivity (resistivity) of the material. Impedance is the opposition to an electric current caused by the resistance of the material. The relationship between these is expressed by:

$$\mathbf{Z} = (\mathbf{R}^2 + \mathbf{Xc}^2)^{0.5}$$

[Equation 2.1]

where Z is impedance (ohm), R is resistance (ohm) and Xc is reactance (ohm).

Resistance is a function of the resistivity, cross-sectional area and length of the conductor as well as the frequency of the applied alternating electrical current. Reactance is a function of inductive resistance and measures the dielectric properties of the conductor (Nyboer 1972).

The relationship between the volume of a conductor and the measured impedance is expressed by equation 2.2:

$$\mathbf{V} \propto \frac{\mathbf{l}^2}{\mathbf{Z}}$$

[Equation 2.2]

where *V* is volume, *l* is length and *z* is the measured impedance.

It follows for equation 2.3 that:

$$V = \rho \times \frac{l^2}{Z}$$

[Equation 2.3]

where ρ is the specific resistivity of the conductive volume. Using equation 2.3 it is possible to calculate volume if l, z and ρ are known.

If this idea is expanded to the human body, current flows preferentially through the areas of lower resistance such as the high water containing FFM – TBW rather than through adipose tissue which

is low in water. Thus equation 2.3 becomes

$$V_{TBW} = \rho \times \frac{l^2}{Z}$$

[Equation 2.4]

as current flows only through TBW at high, theoretically infinite frequencies. This is when the capacitive nature of cell membranes breaks down. Thus equation 2.4 becomes equation 2.5:

$$V_{TBW} = \rho \times \frac{l^2}{R_\infty}$$

[Equation 2.5]

as at infinite frequency, Z and R are identical since there is no reactance.

Conversely at low frequency, theoretically zero frequency, current cannot cross the cell membrane and flows only through the extracellular water as shown in equation 2.6:

$$V_{ECW} = \rho \times \frac{I^2}{R_0}$$

[Equation 2.6]

BIS is the application of an alternating current to the body over a range of frequencies that allows estimation, by extrapolation, of R_∞ and R_0. Equations 2.5 and 2.6 can then be used to determine the volume of TBW and ECW, respectively. Assuming a hydration fraction for FFM allows FFM to be calculated from TBW.

In practice an impedance spectrometer applies an extremely low (<200 μA) current, roughly equivalent to holding the positive and negative ends of a double A (AA) battery between your fingers, and is harmless and imperceptible. BIS is not affected by other monitoring systems, such as ECG to which the infant may be connected (Lingwood, Dunster & Ward 2005).

Four electrodes in total are fitted to the patient, at the palm, wrist, ankle and foot. A weak alternating current is passed through the outer pair of electrodes (wrist and ankle) while the voltage drop across the body is measured using the inner pair of electrodes (palm and foot) from which the body's impedance (resistance to the current) is derived. The impedance at 256 different frequencies ranging from 50 Hz to 1000 Hz is recorded (Figure 2.1).

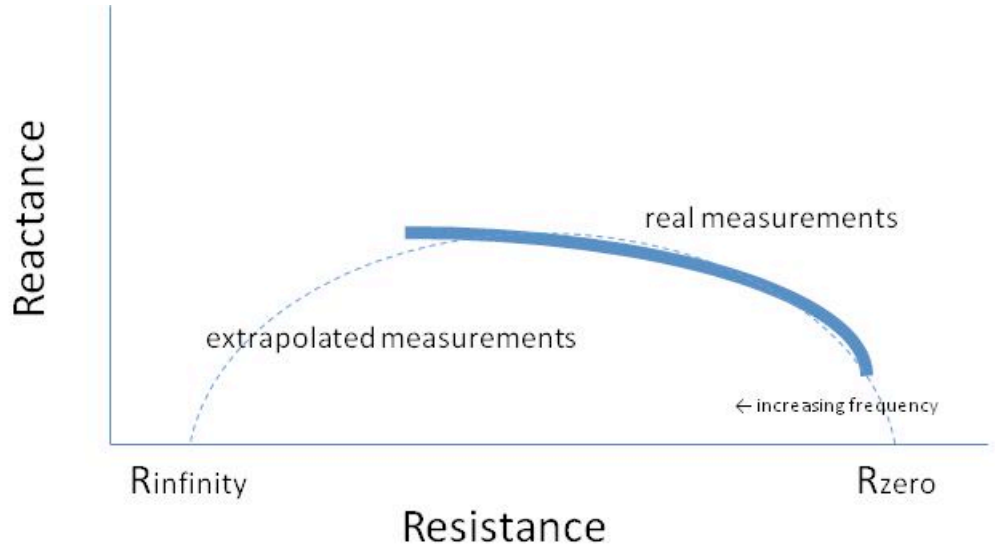

Figure 2.1 Extrapolation of resistance at 0 kHz and at infinite kHz is achieved by plotting measured values of resistance versus reactance on a curve.

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At low frequencies the current can only flow through extracellular water while at higher frequencies it can flow through both extracellular and intracellular water. Resistance at 0 kHz (R_0) best predicts ECW volume and resistance at infinite kHz (R_∞), predicts TBW according to equations 2.5 and 2.6 (Ellis, K. J. 2000). In order to solve equations for TBW and ECW, respectively, values for the resistivities of these fluids (ρ) are required and can be determined empirically. Specific resistivities have been determined in adults but not in preterm infants.

2.3 Trial aim

The aims of this trial were to:

1) Determine resistivity coefficients for ECW and ICW in preterm infants;
2) Cross-validate these coefficients for the prediction of body water in preterm infants; and
3) Evaluate the appropriateness of BIS for the measurement of body composition in nutritional studies in premature infants.

2.3.1 Overview of trial design

TBW and ECW volumes were determined in a cohort of preterm infants by the reference method of tracer dilution using deuterium and sodium bromide (NaBr) for TBW and ECW, respectively. BIS measurements were obtained to allow determination of R_∞ and R_0. Resistivity values were then calculated from equations 2.5 and 2.6.

The predictive value of BIS was then assessed in a separate group of infants using these coefficients to predict TBW and ECW from BIS measurements and to compare these predicted values with those obtained using the reference methods.

This trial has been published in the *European Journal of Clinical Nutrition* (Collins, Carmel Teresa et al. 2013).

2.4 Trial protocol

2.4.1 Participants

Infants were recruited from the neonatal departments of the Women's and Children's Hospital (WCH) and Flinders Medical Centre (FMC) for this cross-sectional validation trial. All infants born and cross-transferred to the neonatal units were screened for eligibility and if successful the mother and/or father were subsequently approached. The initial approach was made by Dr Andrew McPhee (WCH), Dr Scott Morris (FMC) or the neonatal research nurses. A second approach was made by neonatal research nurses to answer questions and hear concerns. Written informed consent was required for the trial to proceed and this was normally obtained by the neonatal research nurses. Ethical approval was granted to this trial on 24 October 2007 after consideration by the Women's and Children's Health Network Human Research Ethics Committee.

2.4.2 Eligibility criteria

2.4.2.1 Inclusion

Infants were eligible if they were born before 37 completed gestation weeks, were greater than one week of age, on full enteral feeds and clinically stable.

2.4.2.2 Exclusion

Infants that were unstable and receiving intravenous fluids were excluded from the trial. An infant was withdrawn from the trial if vomiting occurred during the

equilibrium period as it was impossible to know exactly how much tracer solution remained with the infant.

2.5 Trial methodology

2.5.1 Timeline

A standardised protocol was used with a baseline blood sample taken from the baby. The required amount of tracer solution was calculated and prepared. BIS measurements were recorded, followed by anthropometric measurements. The tracer solution was given to the baby and then the normal feed of either EBM or formula. Three hours after the administration of the trial solution a second blood sample was taken.

2.5.2 Baseline blood sample

A baseline blood sample (0.5 ml, lithium heparin microtainers with no gel separator) was taken by heel prick on the day of the trial by regular laboratory staff at the same time as the infant's routine blood tests were taken. The sample was stored at 4°C.

2.5.3 BIS measurements

The BIS machine (SFB7, ImpediMed Ltd., Queensland, Australia) was calibrated using the test cell each trial day and the date and time confirmed to be correct. A

file name containing the first three letters of the infant's mother's surname and the trial identification number was created and saved on the BIS machine.

The infant remained clothed and in the cot for BIS readings. Four Ag-AgCl electrodes (ImpediMed, Queensland, Australia) were placed on the infant's left side, two on the foot and two on the hand. Electrodes were cut in half due to the small size of the infants. The voltage (sense) electrodes were placed so that the distal edge of the electrode lies along the skin crease on the posterior surface of the wrist (connects to yellow lead) and the ankle at the level of the styloid process and medial malleolus (connects to blue lead), respectively. Current (source) electrodes are placed on the palmar surface of the hand (connected to the red lead) and the planter surface of the foot (connected to black lead) at the distal ends of the metacarpals and metatarsals, respectively (Lingwood et al. 2000). A minimum of two sets of readings were taken, with each reading recording five consecutive frequency scans one second apart. If the infant was seen to touch skin with skin during this time, the reading was repeated. The quality of the reading was checked on the SFB7 display screen (Cornish, Thomas & Ward 1993). If the Cole plot was fitted and was correctly oriented the reading was deemed acceptable. If not, the process was repeated until acceptable recordings were obtained.

2.5.4 Anthropometric measurements

Anthropometric measurements were performed in duplicate by a neonatal research nurse and PhD candidate. The naked infant was weighed using

calibrated neonate scales, to the nearest 5 g. Length was measured as crown-heel using a recumbent length board to the nearest mm (see 3.8 for full description).

2.5.5 Calculation of tracer dose

The target dose of deuterium was 1 ml per kilogram of body weight and 1.5 mmol of NaBr per kilogram of body weight with the exact dose per infant being determined gravimetrically. Deuterium of 99.9% purity (Cambridge Isotope Laboratories, Massachusetts, United States of America) and NaBr of 99.8% purity (Sigma, Missouri, United States of America) were combined in a dose solution as described below.

Solutions were prepared to the nearest 0.001 g by the hospital pharmacy.

Due to institutional regulations preparation of the dilution solution differed slightly between centres. At FMC a stock solution was prepared and transferred by aseptic technique and sterile membrane filtration and refrigerated. At WCH deuterium frozen in 5 ml syringes was thawed and added to the prepared required amount of sodium bromide at the time of administration by a neonatal research nurse.

The amount administered to each infant was determined by the previous day's weight using the calculation: infant weight x 1.154, such that an infant weighing 1.5 kg was to receive 1.73 g of trial solution.

This amount was prescribed on the drug chart by the attending neonatologists and the calculation checked by the neonatal research nurse. The dose to be

administered was then rechecked against a table of ready-made dose weight per kg of body weight. The required amount was drawn up into a syringe which was weighed in duplicate to three decimal places. The prescribed amount of feed was measured out by syringe and 5 ml given via naso-gastro tube. The dosing solution was then given via the tube and the time recorded. The rest of the feed was given as normal. The empty syringe was weighed to three decimal places in order to determine the volume of solution given. A 0.5 ml blood sample was taken three hours later.

2.6 Sample analyses

2.6.1 Pre-analysis

Blood samples were stored at 4°C before being centrifuged using a Sigma 4K15 centrifuge at 300 xg for ten minutes to separate blood into red blood cells, buffy coat and plasma. Plasma was transferred to a clean tube and stored at -80°C.

2.6.2 Transportation

Plasma samples were transferred on dry ice by World Couriers to collaborator A/Professor Leigh Ward at the University of Queensland, St Lucia campus. Upon arrival, the samples were stored at -80°C.

2.6.3 Bromide analysis by High performance liquid chromatography assay

Bromide analysis was performed by the PhD candidate under the supervision of the collaborating researcher at the University of Queensland in Brisbane, Australia according to the method of Miller and colleagues (2014).

High performance liquid chromatography (HPLC) is a laboratory based technique for separating a mixture of compounds and identifying and quantifying the individual components. The sample is forced through a physical or chemical web-like stationary structure with different compounds separating out as they navigate a path.

2.6.3.1 Sample preparation

Plasma samples were left on the bench to thaw and mixed thoroughly using a bench vortex. 50 µl of sample plasma was added to 100 µl of cold acetonitrile in a 500 µl Eppendorf tube. Acetonitrile is a polar solvent and causes the protein in the plasma to precipitate. The Eppendorf tube was immediately vortexed using a bench vortex for 30 seconds and placed in ice for ten minutes. Samples were then centrifuged using a bench centrifuge at 300 g for ten minutes to pellet the precipitated protein. The supernatant (liquid) was carefully removed using a glass pipette from the Eppendorf tube and transferred to a HPLC autosampler tube.

The analyte (sample) is added to the mobile phase and pumped through a column (stationary phase). The speed at which an analyte moves through the column is known as the retention time and is an identifying characteristic of a given analyte.

2.6.3.2 HPLC conditions

The mobile phase was 40 mM KH_2PO_4, pH 3.5 at a flow rate of 1.2 ml/min. The stationary phase was a Phenomenex 250 x 4.6 mm column (10 µM SAX ion exchange, guard column with C18 insert).

The machine was purged at the beginning of each day of testing using the following solutions; 1: water, 2: 50 % v/v water and methanol, 3: 100 % methanol, 4: 50 % v/v water and methanol, 5: water. The detection wavelength was set at 200 nm and the injection volume at 20 µl. The retention times for chlorine and bromide were approximately 4 and 7.5 minutes, respectively. Retention time can be dependent of the size or the charge of the particle and change with different mobile and stationary phases. Bromide reacts more than chlorine in the stationary phase, spending more time there and emerging after chlorine

The retention time for bromide is 7.5 minutes which is detected by UV spectrophotometry. As the analyte leaves the column it passes through a UV light and a detector. The absorption is recorded as a peak on the output screen. The solvents can also absorb UV light and the wavelength set must be higher than that of the solvent to prevent false positive readings.

2.6.3.3 Quantification of bromide

Bromide concentrations in samples were determined from peak heights by reference to a standard curve prepared from known amount of bromide.

2.6.3.4 Calculation of ECW volume

Extracellular water was calculated as corrected bromide space from the quantity of bromide administered and the measured increase in tracer concentration at 3 hours compared to baseline concentration, according to the following equation:

$$\mathbf{ECW} = \frac{\mathbf{Br_{dose}}}{[\mathbf{Br}]_{3h} - [\mathbf{Br}]_{0h}} \mathbf{x\ 0.934\ x\ 0.95\ x\ 0.90}$$

[Equation 2.7]

where 0.934 is the fraction of water in neonate plasma (Hartnoll, Betremieux & Modi 2000), 0.95 is the Donnan equilibrium factor for bromide and 0.9 is the fraction of bromide dose that is retained and assumed to remain extracellular.

2.6.3.5 Deuterium analysis by Fourier Transform Infrared Spectrophotometer

Determination of deuterium concentrations was performed by the research collaborator at the University of Queensland, Brisbane, Australia, using a Fourier transform infrared spectrophotometric method (Tan & Cooke 2008).

Total body water was calculated from the quantity of deuterium administered and the measured increase in tracer concentration at 3 hours compared to baseline concentration according to the following equation:

$$\mathbf{TBW} = \frac{\mathbf{D_2O_{dose}}}{[\mathbf{D_2O}]_{3h} - [\mathbf{D_2O}]_{0h}} \mathbf{x\ 0.934\ x\ 0.96}$$

[Equation 2.8]

where 0.934 is the fraction of water in the neonate plasma and 0.96 is the fraction of exchangeable hydrogen that is assumed to remain with water.

2.6.4 BIS analysis

BIS data were analysed using Bioimp software (v4.12.0.0) provided by the manufacturer (ImpediMed Ltd.). As a quality control measure, BIS data files were cleaned in accordance with preset parameters (Table 2.1), using STATA software (StataCorp, Texas, USA). The values for reactance co-ordinate of the locus of the centre of the fitted semi-circle were required to be negative, the ratio of R∞ to R0 was required to be equal to or greater than 0.2, the values for SEE were required to be equal to or less than 5% and all other values were required to be any number barring 0 to be accepted.

Table 2.1 Data cleaning parameters for raw BIS files

Parameter	Accept	Reject
Xc	Negative number	≥0
*Ratio of $R_{infinity}$ to R_{zero}	≥0.2	<0.2
SEE	≤5%	>5%
Any column		0

Any values for R_0, R_∞, R_i, or the calculated resistance of the intracellular water that lay two standard deviations from the mean were excluded (n=101) from the data set leaving 2325 individual data files to be analysed.

2.6.4.1 Prediction of body water volumes

Body water volumes were predicted using the equations 2.9 and 2.10 from Hanai mixture theory. The extrapolated values of resistance at zero frequency (R_0) and at infinite frequency ($R\infty$) as described in equations 2.5 and 2.6:,

$$\mathbf{ECW} = \frac{\mathbf{1}}{\mathbf{10}} \left(\frac{\rho\mathbf{ECW} \times \mathbf{Kb} \cdot \sqrt{\mathbf{W}} \times \mathbf{L}^2}{\sqrt{\mathbf{D}} \cdot \mathbf{R_0}} \right)^{\frac{2}{3}}$$

[Equation 2.9]

where ρ_{ECW} is the apparent resistivity of the ECW, W is body weight (kg), L is length (cm), D is body density (g/L), R_0 is the resistance at zero frequency and Kb is a body proportion factor. The proportion factor accounts for the different cylindrical geometries of the arm, trunk and leg when making whole body BIS measurements. Kb was calculated as 3.78 using software provided by the manufacturer and from literature values of limb and body proportions for infants of a similar age (Costa-Orvay et al. 2011; Merlob, Sivan & Reisner 1984, 1986; Sivan, Merlob & Reisner 1983a, 1983b). Age-dependent body density values were taken from Fomon and colleagues (1997) and varied from 1.063 to 1.065, giving a mean of 1.064 that was independent of sex and was used in all calculations. Intracellular water was predicted using equation 2.10 and equation 2.11:

$$\mathbf{ICW} = \mathbf{ECW} \left(\left[\frac{\rho\mathbf{TBW} \cdot \mathbf{R_0}}{\rho\mathbf{ECW} \cdot \mathbf{R_\infty}} \right]^{\frac{2}{3}} - \mathbf{1} \right)$$

[Equation 2.10]

where ρTBW is given by:

$$\rho TBW = \rho ICW - (\rho ICW - \rho ECW) \cdot \left(\frac{R_\infty}{R_0}\right)^{\frac{2}{3}}$$

[Equation 2.11]

and ρECW is the apparent resistivity of the ECW, ρTBW is the apparent resistivity of TBW, ρICW is the apparent ICW resistivity and R∞ is the resistance at infinite frequency.

2.6.4.2 Calculation of apparent resistivity coefficients

Sex-specific resistivity coefficients were calculated using infant length, weight Kb, R0, R∞, TBW (from deuterium dilution) and ECW (from sodium bromide dilution) using the resistivity calculator module in Bioimp (v4.12.0.0, ImpediMed, Brisbane, Australia). This module calculates ρICW, ρECW and ρTBW from rearrangement of equations 2.10 and 2.11.

2.6.5 Statistical analyses

Group differences in characteristics between sexes were assessed using group t-tests and by sex and group analysis of variance for cross-validation cohorts. In order to assess the predictive performance of BIS using the newly derived resistivity coefficients a split sample approach was taken.

The population was divided using a random number generator into 1/3, to be used as a validation group and 2/3 to be used as a prediction group whilst maintaining an equal male to female ratio in each group. Data of the prediction group were used to generate resistivity coefficient values as described above. These were

then used in equations 2.10 and 2.11 to predict the TBW and ECW in the validation group. The agreement between the predicted TBW and ECW volumes and the dilution measured volumes in the validation group were assessed by Pearson's and concordance correlations, and limits of agreements analysis. The difference between measured and predicted water volumes in the validation cohort was assessed using a paired t-test.

2.7 Results

2.7.1 Participant characteristics

Ninety nine preterm infants were recruited from the neonatal units of the WCH (n=61) and FMC (n=38). Complete data were available for 91 infants; males n=46, females n=45. Due to vomiting during the equilibrium period four infants were excluded. A further four infants were excluded due to insufficient blood collected for analysis. Clinical baseline characteristics along with TBW and ECW as derived from the dilution methods are presented in Table 2.2.

Table 2.2 Clinical characteristics of infants

	Males n = 46	Females n = 45
Gestational age at birth (weeks)	33.1 ± 2.7	31.9 ± 3.2
Birth weight (g)	1883 ± 542	1681 ± 531
Age at trial (week)	3.2 ± 2.6	3.8 ± 2.1
Post menstrual age at trial (weeks)	35.7 ± 1.8	35.7 ± 1.3
Weight at trial (g)	2239 ± 412	2149 ± 387
Length at trial (cm)	45.0 ± 2.7	44.1 ± 2.3
TBW (ml)	1812 ± 370	1707 ± 301
ECW (ml)	1062 ± 259	987 ± 227

Mean ± SD

When the infants were split into the prediction and validation groups there were no differences between baseline variables (Table 2.3).

2.7.2 Cross-validation between predicted and measured body water volumes

Resistivity coefficients for ECW and ICW are show in Table 2.4. No significant differences were observed between males and females or between predictions and validation groups. The apparent resistivity of ICW was significantly greater than that of ECW. A high degree of variability between individual infants in resistivity coefficients (up to 30% ± SD) was observed.

The group mean predicted volume for TBW was 10 ml greater than measured volumes though this difference was not significant.

Table 2.3 Characteristics of cross-validation group

	Prediction Group		Validation Group	
	Males n = 31	Females n = 30	Males n = 15	Females n = 15
Gestational age at birth (weeks)	33.4 ± 2.7	31.9 ± 3.7	32.4 ± 2.5	32.1 ± 2.0
Birth weight (g)	1926 ± 577	1622 ± 561	1794 ± 467	1795 ± 461
Age at trial (week)	2.9 ± 1.7	4.3 ± 3.3	3.1 ± 1.6	3.5 ± 1.7
Weight at trial (g)	2256 ± 422	2125 ± 343	2201 ± 403	2198 ± 472
Length at trial (cm)	45.1 ± 2.9	44.0 ± 2.0	44.6 ± 2.5	44.5 ± 2.9
Volume of feed (ml)	52.6 ± 18.7	47.5 ± 13.9	51.6 ± 15.6	48.1 ± 15.2
TBW (ml)	1807 ± 384	1673 ± 272	1818 ± 354	1776 ± 352
ECW (ml)	1068 ± 262	992 ± 222	1051 ± 261	976 ± 246

Mean ± SD

There was a very strong correlation between predicted and measured TBW values (Pearson's r=0.825, Table 2.5, Figure 2.2). A reduced concordance correlation coefficient (concordance r=0.742, Table 2.5) suggests that the relationship between measured and predicted TBW values differ from the line of identity. The limit of agreement analysis shows a bias of only 10 ml but with large limits of agreement (± 650 ml, Table 2.5, Figure 2.3).

Mean predicted ECW was 40 ml greater than the measured volume. However, this difference was not significant.

A similar pattern was also apparent with ECW. Predicted volumes correlated well with measured volumes (r=0.75, Table 2.5, Figure 2.4), with the relationship differing from the line of identity (r=0.743, Table 5). The limits of agreement analysis showed a bias of 40 ml with again, large limits of agreement (± 360ml, Table 2.5, Figure 2.5).

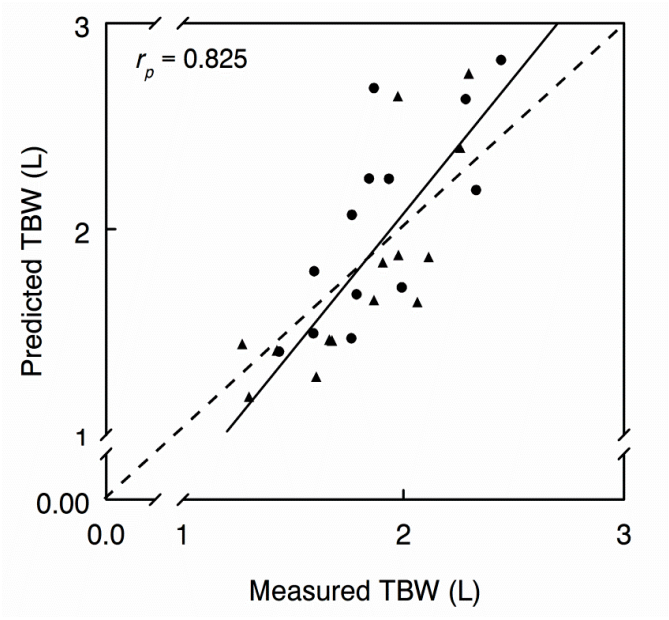
Table 2.4 Resistivity coefficients for prediction participants, validation participants and all participants

	Resistivity coefficients (ohm/cm)	
	ECW	ICW
Prediction participants		
Males (n=31)	640.2 ± 162.0	1405.3 ± 452.3
Females (n=30)	693.0 ± 172.5	1556.1 ± 450.6
Validation participants		
Males (n=15)	662.6 ± 237.3	1483.9 ± 715.2
Females (n=15)	653.6 ± 203.8	1682.0 ± 477.7
All participants		
Males (n=46)	645.7 ± 185.4	1426.5 ± 536.4
Females (n=45)	678.4 ± 181.3	1593.9 ± 453.6

Mean ± SD

Table 2.5 Measured and predicted body water values in validation group using resistivities derived from the predicted group

	Body water compartment	
	TBW	ECW
Measured (L) mean ± SD	1.79 ± 0.34	1.01 ± 0.26
Predicted (L) mean ± SD	1.80 ± 0.55	1.05 ± 0.26
Pearson correlation coefficient (r_p)	0.825	0.750
Concordance correlation coefficient (r_c)	0.742	0.743
Bias (L)	-0.01	-0.04
Limits of agreement (2 SD) (L)	0.64, -0.66	0.32, -0.39



F

Figure 2.2 Correlation of predicted and measured TBW in the validation group. ♦ females; •, males; ———, fitted regression line; – – –, line of identity.

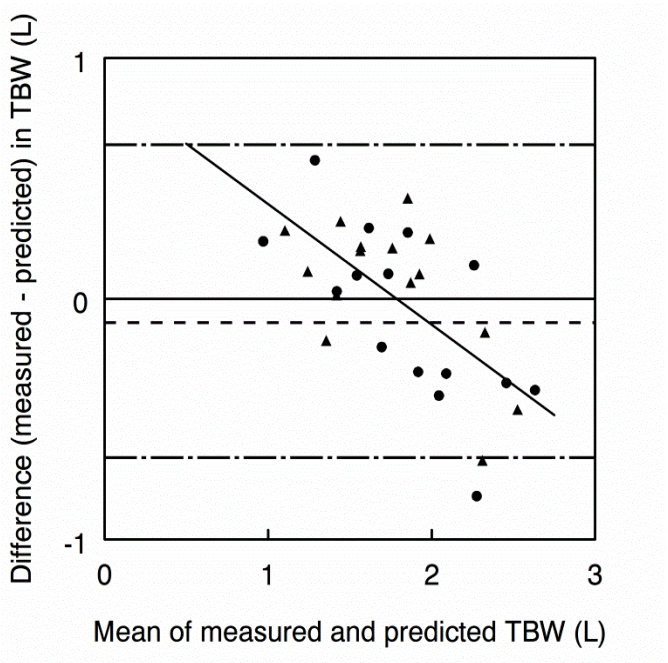


Figure 2.3 Bias and limits of agreement between predicted and measured TBW in the validation group. ♦, females; •, males; ———, fitted regression line; – – –, bias; -- - --, two SD. limits of agreement.

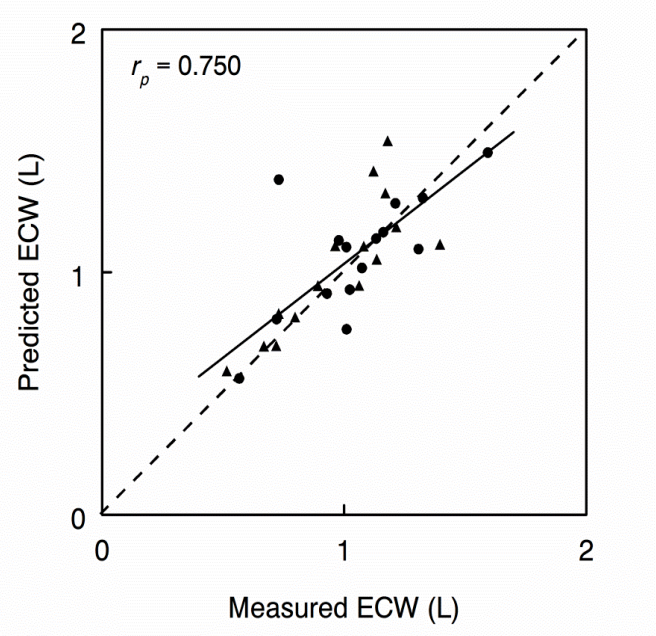


Figure 2.4 Correlation of predicted and measured ECW in the validation group. ♦ females; •, males; ———, fitted regression line; – – –, line of identity.

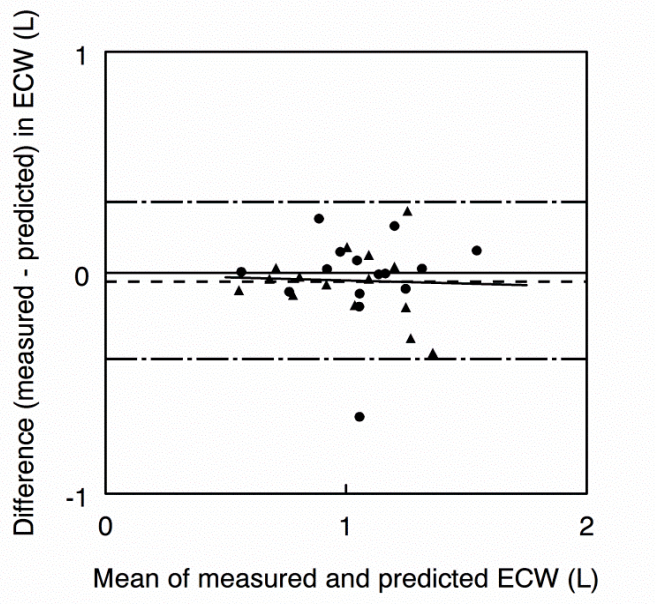


Figure 2.5 Bias and limits of agreement between predicted and measured ECW in the validation group. ♦, females; •, males; ———, fitted regression line; – – –, bias; -- - --, two SD. limits of agreement.

On the basis of this close agreement, resistivity coefficients were calculated for the validated subjects and provided values very similar to those observed in the prediction group. There was no difference between the values; therefore data from both groups were combined to produce overall estimates for resistivity coefficients.

2.8 Discussion

This trial successfully established resistivity coefficients for preterm infants using the gold standard methods for determination of water volumes as reference. The use of population-specific resistivity coefficients and population level Kb values provided good predictive power for TBW and ECW. The mean biases were 10 and 40 ml for TBW and ECW, respectively. These represent errors of 0.56 and 3.96%. However, the magnitude of the limit of agreement was much larger, approximately 35%, indicating that BIS cannot provide an accurate measure of the body composition of an individual preterm infant and is not therefore suitable for estimating absolute fluid volumes in a clinical setting. However, despite this weak accuracy at an individual level, the precision of BIS is high. This suggests that BIS would be useful at looking at changes in body water values over time even in an individual subject where the importance is in the overall trend and less about absolute accurate numerical values. The small biases also indicate that BIS may be useful for measurements between populations or for comparisons between groups within a population, e.g. before and after treatment intervention, where the population means could be compared.

The reasons for the poor predictive nature of BIS in infants are unclear however there are several factors to be considered. Owing to the indirect method of measurement and the various assumptions applied in equations 2.7 to 2.11, errors are propagated throughout the calculations to the final predicted value. Errors are present in the reference techniques, body proportions, body density, body weight, body length and resistivity coefficients. Similarly, biological factors due to the nature of prematurity can induce error. Preterm infants are in a rapidly changing physiological state. Body water as a component of body weight is much higher than in adults. It is also highly variable and presents as a much higher ECW proportion than adults. Body water volumes and the relative proportions of ECW to ICW are rapidly changing impacting on the accuracy of equations 2.10 and 2.11.

Despite the limitations of BIS in preterm infants, it is still a valuable tool for research. While the lack of absolute accuracy of the BIS method rules out using individual measurements for preterm infants, the high degree of precision and very strong correlations between the predicted and measured body water volumes in the validation trial show that the method has value where the primary goal is looking at changes in body water volumes over time between groups.

Methods of a randomised controlled trial of increased protein fortification of breast milk

Chapter 3 Methods of a randomised controlled trial of increased protein fortification of breast milk

The candidate was responsible for the entire conduct of this randomised controlled trial including protocol development, ethics submissions, recruitment, randomisation, infant measures, daily study product ordering, sample storage, data collection and management, analysis of milk samples using the MilkoScan Minor, creation of the data analysis plan and analysis of demographic data and interpretation of study results.

This chapter describes the design and conduct of a randomised controlled trial assessing increased protein fortification of EBM in preterm infants born 28–32 weeks gestation. The trial compared growth of infants receiving fortifier with an additional 0.8 g protein/100 ml EBM over the current clinical practice fortification of 1.0 g protein/100 ml EBM (total of 1.8 g protein/100 ml EBM). The fortifier was given as an entrée (before EBM) supplement in both groups. By providing the supplement as an entrée before all feeds – including direct breast feeds – were supplemented. This novel, pragmatic trial was designed to test the efficacy of both increasing protein content compared to standard practice and allowing direct breast feeds to be supplemented. The trial was registered with the Australian New Zealand Clinical Trials Registry (ANZCTR) before recruitment began (www.ANZCTR.org.au/ACTRN12611001275954.aspx). The trial was known in the nursery as Poppet (Providing optimal protein for prems via enteral tubes).

3.1 Objectives and hypotheses

The objectives of this trial were to increase the protein content of HMF to levels that met international recommendations (while maintaining the same energy level) and to ensure direct breast feeds could be fortified by administering the HMF separately from breast milk.

3.1.1 Primary hypothesis

Preterm infants fed a higher level of protein HMF entrée (1.8 g/100 mL EBM) before breast milk feeds will have improved weight gain (g/week) when compared with infants fed breast milk fortified at standard levels (1.0 g/100 mL).

3.1.2 Secondary hypothesis

Preterm infants fed a high protein HMF entrée before breast milk feeds (when compared with a standard protein HMF entrée) will have:

- Improved length gain (cm/week)
- Improved head circumference gain (cm/week)
- Improved lean mass accretion (lean mass/body weight), and
- Lower likelihood of being classified SGA for weight at discharge.

3.2 Safety outcomes

In a previous protein supplemented trial in this unit, a urea of < 8.0 mmol/L was determined by the neonatologists to be defined as a uraemia, for the purpose ofs clinical notification and review (Miller et al. 2012). Eleveated urea has been used as a marker of protein intake and tolerance in other clinical trials (Arslanoglu, Moro & Ziegler 2006).

3.3 Trial design

This trial was a single centre randomised controlled trial with parallel group design.

3.4 Ethical approval

Conditional ethical approval was granted to this trial on 3 November 2011 after consideration by the Women's and Children's Health Network Human Research Ethics Committee. Full ethical approval was granted on 25 November 2011. Ethical approval for infants that had been enrolled at the Women's and Children's Hospital (WCH) and down transferred to the Lyell McEwin Hospital was granted on 15 May 2012 by the Human Research Ethics Committee (The Queen Elizabeth Hospital/Lyell McEwin Hospital/Modbury Hospital) with support from the acting Medical Director and Clinical Services Coordinator of the Lyell McEwin Hospital neonatal services.

3.5 Participants

3.5.1 Settings and location

Recruitment for the trial took place at the WCH, North Adelaide, Australia. The WCH is the largest perinatal tertiary centre in South Australia. The Neonatal Unit consists of a 14 bed Neonatal Intensive Care Unit (NICU) (Level 6) and a 35 bed Special Care Baby Unit (SCBU) (Level 5) staffed by 5.8 full time equivalent consultant neonatologists, 2 fellows, 7 registrars and 3.6 neonatal nurse practitioners. While infants can be down transferred to a number of Level 4 or Level 3 neonatal units in South Australia, parents were only approached if anticipated down transfer was to the Lyell McEwin Hospital (Level 5) for pragmatic reasons relating to trial management.

3.5.2 Eligibility criteria

3.5.2.1 Inclusion criteria

Infants were eligible if born 28 to 32 completed weeks gestation, their mothers intended to provide breast milk and parents or guardians provided written informed consent.

Infants less than 28 weeks gestation are at high risk of growth failure. The WCH clinical practice dictates that if growth failure occurs and BUN levels are <2 mmol/L many of these very preterm infants have their fortified feeds supplemented with additional protein. To ensure that the trial intervention was the only difference

between the groups, only infants 28 completed weeks gestation or older would be eligible.

It was important that the infants were in-patients for enough time for the intervention to affect growth rate. Preterm infants normally remain in hospital to term or close to, so it was reasonable to assume that infants of 32 weeks gestation would be in hospital for three to four weeks.

3.5.2.2 Exclusion criteria

Infants with major congenital or chromosomal abnormalities known to affect growth or where protein therapy was contraindicated (e.g. major heart defects, cystic fibrosis, phenylketonuria, disorders of the urea cycle) were excluded. Infants likely to transfer to remote locations and infants that had received standard fortifier for more than four days were also excluded.

3.5.3 Recruitment

All infants in the NICU and SCBU were screened by neonatal research nurses for eligibility (Appendix B). When the infant was stable, well and approaching introduction of fortified feeds, parents of eligible infants were given verbal and written information about the trial by a neonatal consultant or a neonatal research nurse (Appendix C). Parents were given time to consider their participation in the trial and to discuss with family members or staff. Follow up for consent was

performed by the neonatal nurses that do not provide direct clinical care, removing the potentially dependent relationship between clinicians and patient (Appendix D).

3.6 Randomisation

3.6.1 Sequence generation

The randomisation schedule was computer generated using variable block design (4, 6). Stratification occurred for sex and GA <29 weeks and 30 to 32 weeks. Multiple births were randomised as individual infants, with the infants randomised according to birth order.

3.6.2 Allocation concealment mechanism

The research team, health care team, and parents of the infant were unable or permitted to influence the allocation procedure or predict the assignment.

The allocation process was conducted by telephone to an independent researcher that held the randomisation schedule.

3.6.3 Implementation

All infants whose parent/guardian gave consent for participation and that fulfilled the inclusion criteria were randomised. An independent biostatistician (Professor Philip Ryan) developed the randomisation schedule and an independent

researcher held the randomisation schedule. The allocation process was conducted by telephone to the independent researcher as follows:

- A phone call was made to a research support staff member that was independent of the recruitment process and all other aspects of the trial. A back up staff member was nominated if the primary staff member was unavailable.
- Eligibility criteria were confirmed and the independent researcher confirmed the strata group for that infant.
- The randomisation schedule was consulted and the trial ID and group colour from the appropriate stratum allocated.
- This information was recorded on the allocation log, case report form (CRF), the medical notes for the infant, daily care plan of the infant and daily Poppet order list.

3.7 Interventions

The infants were randomised to one of two groups:

1) High protein group: Infants in this group received a human milk fortifier, FM85 (Nestle) providing 1.0 g protein/100 ml EBM with Protifar (Nutricia) added to provide a total of 1.8 g protein/ 100 ml EBM (see Appendix E for nutritional content). Protifar is a concentrated bovine milk protein, predominantly casein.

2) Standard protein group: Infants in this group received a human milk fortifier, FM85 (Nestle) equivalent to standard care to provide 1.0 g protein/ 100 ml expressed breast milk.

3.7.1 Balancing the energy intakes

As energy concentration is known to influence growth, it was important to ensure that the solutions were isocaloric with a similar nutrient profile. The trial solutions were based on a currently available commercial human milk fortifier (FM85, Nestle). By adding either a bovine milk based protein powder (Protifar, Nutricia) or a glucose polymer powder (Polyjoule, Nutricia), trial solutions were produced that differed only in their protein content. Both Protifar and Polyjoule are nutrition support products commonly used in a neonatal care setting. Glucose polymers such as Polyjoule are already an ingredient in commercial HMF. Neonatologists and dieticians currently supplement the feeds of preterm infants with Protifar or Polyjoule on an ‘as needs’ basis.

3.7.2 Administration of intervention

In standard clinical practice, fortifier is mixed with EBM before it is administered to the infant. Infant nutrition attendants fortify a 24 hour supply of EBM each day and deliver it to the nursery refrigerators. The fortified EBM is then fed to the infant via the nasogastric tube or bottle. In this trial a novel means of administration was implemented in that infants in both groups received an ‘entrée’ of fortifier. The entrée comprised the fortifier and additional protein or glucose powder mixed with

sterile water. The scheduled feed was then given immediately following the entrée, which was administered via the naso-gastric tube.

Under current clinical practice direct breastfeeds are encouraged, however this means that the feed cannot be fortified. The intervention used in this trial allowed direct breastfeeds to be fortified ensuring all feeds are fortified. When the infant received a direct breast feed, the timing of the administration of the trial product (before, during or after the feed) was at the discretion of the primary care nurse in consultation with the mother.

The milk supply of the mother does not always match the demand of the infant. When this occurred the nursery staff prepared a feed using all the available EBM and made up the difference with formula. When the infant received a mixed feed, that is part EBM and part formula, the Poppet entrée was only given if EBM was more than 50% of the total feed as formula already meets the recommended nutritional intake for preterm infants.

3.7.3 Concomitant care

All infants received clinical care and management according to the neonatal unit policies and procedures under the direction of the attending neonatologist with the only difference being the trial fortifier.

3.7.4 Criteria for discontinuing allocated intervention

After discussion with neonatologists, uraemia was defined as BUN levels greater than 8.0 mmol/L for the purposes of this trial.

Infants on full enteral feeds that developed a BUN >8.0 mmol/L and/or a metabolic acidosis (base excess <-6 mmol/L) had the BUN and blood gas analysis repeated within 48 hours and the attending clinician was notified.

If the BUN remained elevated and:

1) was associated with a base excess of <-6 mmol/L then the trial fortifier was discontinued and infant commenced on S26-SMA fortifier for 48 hours and was managed as clinically indicated. S26-SMA was the fortifier used routinely by the nurseries at the time of the trial and contains 1g protein/100 ml. If the acidosis was corrected, the infant continued in the trial and blood tests were monitored twice weekly for one week.

2) was not associated with other abnormal blood tests the infant remained in the trial with BUN and chemistries monitored twice weekly until return to normal.

3.7.5 Criteria for adding additional protein

Infants that had poor weight gain (defined as <15 g/kg/day over the preceding 7 day period) associated with a BUN of <2 mmol/L, once full enteral feeds had been reached, were assessed by the attending neonatologist. Feeds were increased

from 170 to 180 ml/kg/day. If weight gain did not improve, additional protein supplements (Protifar) were added at the discretion of the attending neonatologist, while the infant continued to receive the allocated intervention fortifier. The additional protein was ceased and when weight gain of 15 g/kg/day was achieved and BUN levels measured ≥2 mmol/L.

3.7.6 Participant withdrawal request

If parents wished to withdraw their infant from the trial, the infants received standard fortifier delivered in the standard format. Verbal permission was sought to continue collecting anthropometric and intake data.

3.7.7 Education and support of care staff

Nurses and midwives were provided with detailed instructions on the significance of the trial, the daily tasks involved and the importance of following the trial protocol during a series of workshops A two page instruction sheet was clipped to each infant's care plan for cot side clarification. The PhD candidate was contactable at all times by phone and pager and was 'on call' for the entirety of the study period. The candidate regularly visited the nursery for weekly measurements and Poppet dose orders, and was able to form good working relationships with the staff. When new care staff members were employed during the trial period, the candidate approached them individually to inform them of the protocol and answer any questions or concerns.

3.7.8 Calculation and ordering of Poppet dose

3.7.8.1 High protein fortifier

5 g HMF (1 g protein) and 0.9 g Protifar (0.8 g protein) was mixed with 4 mls of sterile water. This resulted in a total of 8 mls of solution containing 1.8 g protein. (Table 3.1 Trial solutions recipe). This was delivered at 8 mls Poppet fortifier per 100 mls EBM (1.8 g protein/100 mls EBM).

3.7.8.2 Standard protein fortifier

5 g HMF (1 g protein) and 0.9 g Polyjoule (0 g protein) was mixed with 4 mls of water. This displaced to a total of 8 mls solution containing 1.0 g protein (Table 3.1 Trial solutions recipe). This was delivered at 8 mls Poppet fortifier per 100 mls EBM (1 g protein/100 mls EBM). The mean osmolarity of the study solution at this concentration was 485 mmol/kg.

Table 3.1 Trial solutions recipe

Poppet entrée	Water mls	HMF (FM85) g	Protifar g	PolyJoule g	Total volume mls	Total protein g /100 EBM	Total calories kcal/100 EBM
High Protein	4	5	0.9	-	8	1.8	18
Standard Protein	4	5	-	0.9	8	1.0	18

A ‘medication’ label was placed on the medication chart and the fortifier calculated and ordered by the medical or neonatal nurse practitioner. For example, the calculation for an infant weighing 1.2 kg required a total enteral intake of 160 ml/kg/day and fed 3 hourly (8 feeds a day) is as follows:

1) Calculate total intake (mls/day):

1.2 kg x 160 ml/kg/day = 192 mls

2) Calculate total fortifier required (mls/day) where fortifier is to be delivered at 8 mls Poppet solution/100 mls EBM:

(8 x 192) ÷ 100 = 15.4 mls

3) Calculate fortifier required per feed (mls/feed):

15.4 ÷ 8 = 1.9 mls

The medical or neonatal nurse practitioner would order the fortifier on the medication chart as shown in Figure 3.1.

POPPET REC 2401 COLOUR Date:	Weight (kg) **1.2**	ml/kg/day **160**
	mls/day **192**	
	mls fortifier *(0.08 x mls/day)* **15.4**	
	mls fortifier/feed **1.9**	
Dose Frequency **1.9 mls** **3 hourly with feeds**		
Prescriber Signature Print Name		

Figure 3.1 Medication labels used to order Poppet fortified (worked example)

Fortification was to begin at an enteral intake of 80 ml/kg/day on the attending consultant's orders. The Poppet dose was recalculated twice weekly according to current weight and intake, then checked and prescribed by a neonatologist.

3.7.9 Preparation of Poppet trial fortifier

Specialised, trained infant nutrition attendants were provided with a standard operating procedure (SOP) (Appendix F) for making Poppet fortifier and were required to attend training sessions until assessed as competent. The department conducted regular audits of Poppet preparation. The infant nutrition attendants were required to keep detailed records of Poppet tin number, batch number of FM-85, date tin was opened and product expiry date.

Poppet fortifier was made up in the diet kitchen, located on a separate floor to the nursery. The attendants prepared each colour group in its entirety. The attendants weighed FM-85 HMF and Poppet trial powder on scales weighing to 0.1 g accuracy (calibrated annually). Sterile water was measured in a jug and added to the mixing container. The container was shaken by hand for a minimum of two minutes and continued until the attendant was satisfied with the consistency of the mixture and that all powder was dissolved into the water.

The product was decanted into ‘serving size’ syringes with a specialised label attached detailing infant identification, volume and trial details in accordance with TGA (Therapeutic Goods Association) guidelines. Poppet syringes were taken to the neonatal unit fridge where each infant had an individually labelled container. Any syringes that were not administered in the 24 hour period were recorded and discarded.

A daily list which detailed infant identifiers (family name, mother’s name, unit registration number), total ml of Poppet required, number of syringes required,

amount in each syringe required and randomisation colour) was emailed to the infant nutrition attendants each afternoon for preparation the next morning.

3.7.10 Blinding

Four colours (red, green, yellow, purple) were assigned to the trial product. Two of the colours were the intervention arm and the remaining two the control arm. All research staff (including statisticians), clinical staff, participants and their families were blind to allocation. The blind was only broken on completion of the primary and secondary outcome analyses.

Protifar (intervention arm) and PolyJoule (control arm) were packaged in identical 400 gram cans with a 12 month shelf life (Pharmaceutical Packaging Professionals Pty Ltd, Thebarton, Australia). A tamper proof seal was placed across the lid of each tin. Tins were discarded 28 days after opening. The tins were differentiated by colour-coded labels.

The tins were labelled according to TGA guidelines, including the trial colour, amount of product in the tin, the possible contents of the tin, the Research Ethics Committee approval identifier for the trial (2401/10/14), chief investigator contact details, instructions for use, expiry date, batch number and the direction ‘For clinical trial use only, keep out of reach of children’ (see Figure 3.2).

While the infant nutrition attendants were blinded to allocation, the intervention product is visibly different to the control. Protifar is pale yellow, PolyJoule is white. As part of their training attendants were informed as to the importance of blinding

in a clinical trial and were not permitted to speak to the research or clinical staff regarding trial product.

Figure 3.2 Trial product tins labels.

3.7.11 Monitoring nursery compliance

Administration of the Poppet fortifier against the fluid balance chart was regularly audited for compliance to trial protocol.

3.8 Outcomes and assessment

Trial start was defined as day of randomisation and trial end as the removal of the naso-gastric tube or EDD, whichever occurred first. The PhD candidate was trained in the correct measurement of preterm infants for research by two neonatal research nurses, each with over 30 years of neonatal nursing experience and 10 years of research experience (for measurement SOPs see Appendix G).

3.8.1 Primary outcome – weight gain

The primary outcome of the trial was rate of weight gain (g/week) from trial start to trial end.

Infants were weighed daily on annually calibrated scales measuring to 10 g by clinical staff while in NICU and SCBU. Weight was measured by the PhD candidate and one of two neonatal research nurses at randomisation, then weekly while in NICU and SCBU and at the trial end. The infant was undressed, the scale was tarred and the infant placed on the scale. The infant was then lifted, the scale tarred and the infant placed back on the scale. If the weight differed by 10 g the process was repeated.

3.8.2 Secondary safety outcomes

Feeding tolerance was measured by two variables:

- The number of days on which one or more feeds were stopped
- The number of days taken to reach full enteral feeds (enteral intake ≥150 ml/kg/day).

3.8.2.1 Biochemistry metabolic markers

Protein safety and use were assessed by weekly blood tests (0.5 ml) and were coordinated with routine tests where possible:

- blood urea nitrogen
- serum albumin
- plasma creatinine
- blood pH and base excess
- blood amino acids (blood spot), and
- urinary urea, albumin, creatinine (every two weeks).

3.8.3 Secondary efficacy outcomes

The secondary efficacy outcomes for the trial were length gain (cm/week), head circumference gain (cm/week), small for gestational age characterisation by weight, length, head circumference and lean mass proportion.

3.8.3.1 Length gain.

Weekly, measures of recumbent length were taken in duplicate in the supine position to the nearest 0.1 cm using a recumbent length board by the PhD candidate and a trained neonatal research nurse. The infant was positioned in supine parallel with the head flat against the headboard. The head was positioned with the assistant's left hand on the infant's right temporal bone and the assistant's right hand on the infant's left temporal bone such that the assistant was looking down at the infant's upside down face. The primary measurer stands side on to the infant and the length board. When the infant is deemed to be lying straight on the length board, the knees were gently pushed down with one hand to allow the back of the knee to touch the length board. Both the primary measurer and the assistant watched the infant to ensure that the back was not arched, the shoulders and buttocks touched the board, and that the body remained parallel to the board. When all quality control criteria were met, the footboard was moved to touch the infant's feet. The soles of the feet were flat against the footboard with the toes pointed upwards. The board was pressed firmly to the feet, compressing the soft tissue but not enough to cause extreme discomfort or decrease vertebral column length. The primary assessor noted in the CRF but did not remark out loud the measurement in cm to one decimal place. The process was repeated with the primary measurer and assistant switching positions. The two measurements were then compared. A third measurement was taken if there was a discrepancy of more than 5 mm.

3.8.3.2 Head circumference gain

Weekly head circumference was measured in duplicate by a trained neonatal research nurse. The measurement was taken around the largest occipito-frontal circumference using a non-stretching tape. A third measurement was taken if there was a discrepancy of more than 5 mm. Head circumference growth is associated with later neurodevelopmental scores.

3.8.3.3 Small for gestational age (SGA)

Infants measuring <10th centile at trial end for weight, length or head circumference were designated SGA (Beeby, Bhutap & Taylor 1996).

3.8.3.4 Body composition

Lean body mass was obtained by Bioelectrical Impedance Spectroscopy (BIS). BIS determines lean mass proportion by running a weak current through the infant from electrodes attached to the left hand and foot (for SOP see Appendix H). As electrical current moves faster through lean mass and slower through anhydrous fat mass, the body composition can be extrapolated from the time the current takes to reach the sensing electrode. As the BIS calculations require an exact body weight, the infant was weighed immediately after a BIS reading. See 2.5.3 for measurement procedures, 2.6.4 for data cleaning procedures.

3.8.3.5 Other clinical outcomes

A number of other clinical outcomes that were unlikely to be affected by the intervention were collected: grade of intra-ventricular haemorrhage (IVH), confirmed sepsis, confirmed necrotising enterocolitis, grade of retinopathy of prematurity, days of corticosteroid use and respiratory outcomes.

3.9 Data collection

Data were recorded in the CRF (Appendix I) of the infant using the most appropriate source documents. Daily intake data (EBM intake, number of breast feeds, Poppet intake, number of times Poppet solution was given over number of feeds, formula intake, number of feeds greater than 50% formula, name and caloric strength of formula, if feeds were held, if Protifar, PolyJoule or any other additional supplements were given) was taken from the fluid balance chart. Days of parenteral and intravenous lipid administration were added using the fluid balance charts stored in the case notes.

Daily weight measurements from days the infant was weighed by nursery staff and the day the infant reached full enteral feeds (>150 ml/kg/day) were taken from the growth chart. Mother's date of birth, gravidity, parity and EDD were taken from the birth information sheet stored in the case notes. All other information was collected from the Clinical Information Services (CIS) database. Specially trained nurses and midwives collect neonatal data from the case notes according to local and national data collection forms and data dictionaries. The data collection is of the highest quality and is subject to a rigorous external auditing process. The CIS staff

provided the candidate with training in navigating and interpreting the CIS database. From the CIS database mother's unit registration number, mother's age at time of infant's birth, ethnicity of mother, assisted conception, previous preterm birth, previous preterm death, presenting antenatal problem, administration of antenatal steroids, mode of birth/delivery, plurality, birth order, Apgar scores at one and five minutes, birth weight, birth length, birth head circumference, date of final discharge, discharge weight, discharge length, discharge head circumference, main respiratory diagnosis, inpatient date of final added oxygen, home oxygen, hours of intermittent positive pressure therapy, incidence of high frequency ventilation, hours of continuous positive airway pressure therapy, hours of humidified high flow nasal cannula therapy, nitric oxide, maximum grade of intraventricular haemorrhage, cerebral cystic formation, retinopathy of prematurity, proven necrotising enterocolitis, surgery, postnatal steroids, exogenous surfactant, early onset sepsis, late onset sepsis and chronic lung disease were collected.

The mothers were verbally asked about frequency of smoking cigarettes during pregnancy, units of alcohol consumed during pregnancy, highest level of completed secondary school and highest level of further trial.

Data from weekly blood tests were printed from the South Australian Health Network clinical database (OASIS) and entered directly into an electronic database to eliminate transcription errors. Variables collected were: creatinine, albumin, blood urea nitrogen, base excess, pH, glucose, sodium, potassium, chlorine, calcium.

A weekly sample (8 ml) of breast milk was collected and frozen for later analysis of the macronutrient content of the milk.

A urine sample was collected every two weeks by placing a cotton ball in the infant's nappy (1–2ml). The cotton ball was squeezed into a 30 ml specimen container and stored at -80°C.

In conjunction with the biochemistry blood test, a spot of blood was collected on specialised filter paper. The spot was allowed to air dry and then delivered to the metabolic laboratory for amino acid analysis.

3.10 Sample size

A power calculation performed before recruitment began estimated that 60 infants would allow a detection of weight gain of 3.31 g per day difference between the high protein and standard protein groups at 80% power and p=0.05. Consultation with the neonatal medical team agreed that 3.31 g per day was a clinically important difference on which clinical practice would be changed. Sixty infants was an achievable number of infants to recruit in the timeframe afforded by a PhD.

3.11 Sample analysis – breast milk

3.11.1 Principles of MilkoScan Minor

Briefly, a sample of milk is drawn up into the MilkoScan Minor (Foss) by the sampling pipette. The milk is then heated and homogenised before passing into a

cuvette. Infrared light is guided through the sample and the absorbance recorded by a detector. The amount of light absorbed by the sample is converted to an electrical signal and then using an algorithm to a percentage of components.

3.11.2 Set up

The machine was allowed to warm up and was then ‘zeroed’. A custom setting, calibrated especially for preterm breast milk was selected (Miller 2010). Precision of the calibration was confirmed by analysing three samples of independently tested EBM with a known protein concentration. The high, medium and low sample protein concentrations all fell within two standard deviations of the independently derived values.

3.11.3 Analysis

Frozen samples were thawed in warm water and then vortexed for 30 seconds on a bench vortex to ensure protein and fat concentrations were uniform throughout the sample. The sample was then placed under the drawing up pipette and the analysis button pressed. The sample was then suctioned by the pipette into the MilkoScan Minor.

3.11.4 Sample collection

Sample collection to determine the protein content of breast milk was approached in a pragmatic fashion. Expressed breast milk samples (8 ml) were collected

weekly from all women that consented to the Poppet trial if supply was sufficient. This was determined by the PhD candidate on a week by week basis. Factors taken into consideration when deciding if supply was sufficient included the amount of stored EBM, and if the baby had received any formula. Administration of formula suggests that the demand of the baby was greater than the supply of the mother and a sample of milk was not taken if the infant had received formula feeds that week.

3.11.5 Standardising MilkoScan Minor

Stock samples of low, medium and high protein levels that had previously been independently analysed using the Kjeldahl method (Miller 2010) were thawed and analysed using the MilkoScan Minor. One each of the three standards were analysed on every use of the milk analyser. Quality was assessed using the 2_{3s} rule: i.e. the run was rejected if two of the three controls fell outside of 3 SDs from the mean. All three stock samples returned results within 2 standard deviations of the independent results.

3.11.6 Breast milk sample analysis

Breast milk samples were obtained from 32 of the 45 women involved in Poppet, ranging from a single sample to eight samples taken a week apart. All gestation ages included in the Poppet trial were represented. There was a high proportion of multiple birth mothers (9/14) that were able to supply milk for analysis.

A total of 114 samples were obtained and stored at -80°C. Of the 114 samples, 113 gave appropriate readings with one sample of insufficient volume to accurately analyse. This sample was omitted from further analysis.

3.11.7 Mean protein concentration

The mean protein in expressed breast milk of mothers of preterm infants was 1.38 ± 0.37 g/100ml and the mean fat content was 3.87 ± 0.96 g/100ml. The MilkoScan Minor is unable to give accurate carbohydrate results and as such they will not be published in this thesis.

There were large inter and intra-woman differences in protein content of expressed breast milk. The highest content of protein was 3.29 g/100 ml (sample collected 18 days post-partum) and the lowest was 0.79 g/100 ml collected 64 days postpartum (Figure 3.3).

As expected, the protein content of expressed breast milk decreased as days post-partum increased (Figure 3.4).

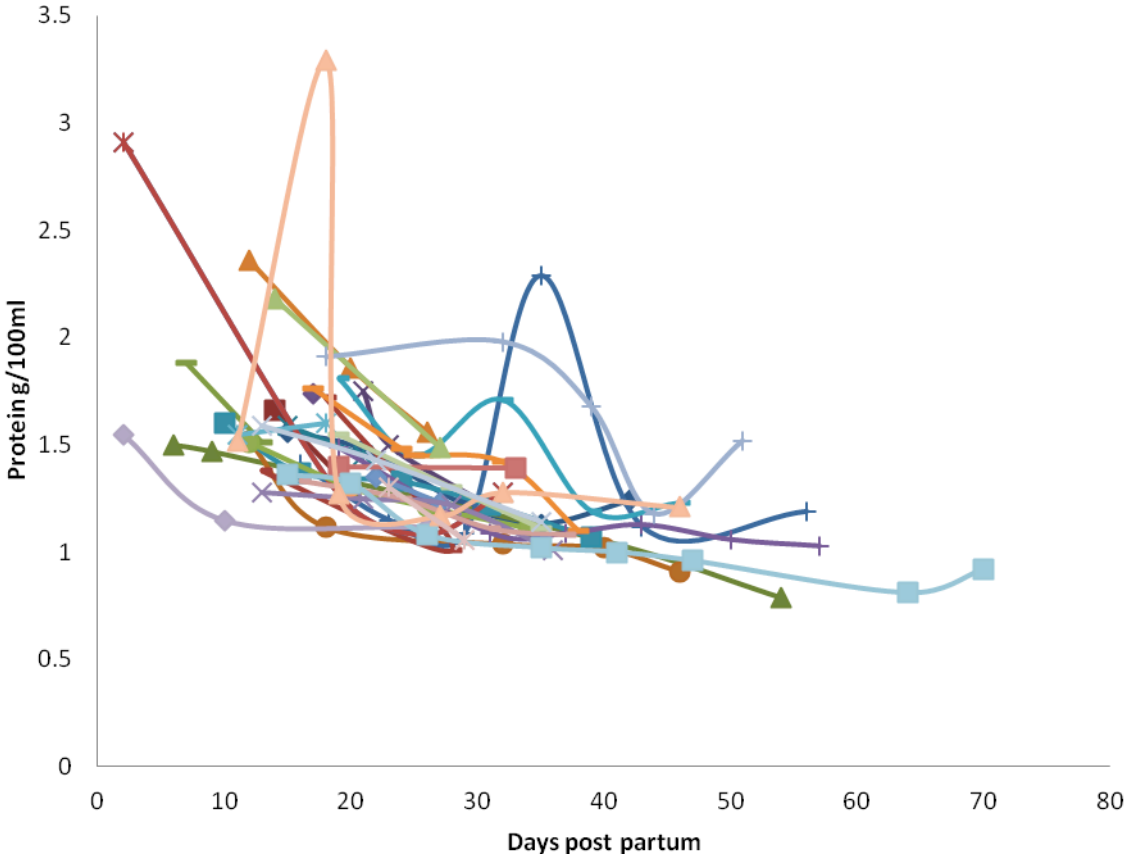


Figure 3.3 Protein content of breast milk from each mother by days post partum.

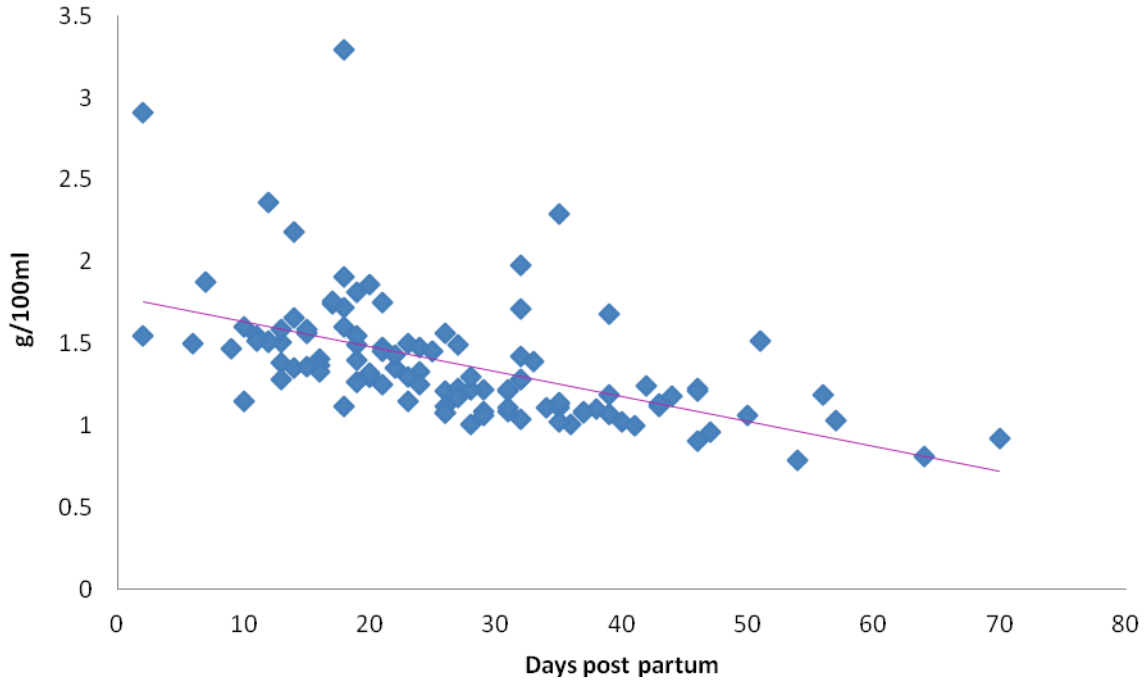


Figure 3.4 Protein concentration of EBM decreases as days post-partum increases

3.12 Sample analysis – blood spot

Blood spots on filter paper from heel prick (3 mm) were collected weekly, allowed to dry and analysed by South Australia Pathology, Neonatal Screening Centre, WCH, using tandem mass spectromony (API4000 triple-quadrupole). A stable isotope dilution technique was used to measure the quantities of amino acids and acylcarnitines. (Naylor & Chace 1999; Rashed et al. 1997).

3.13 Sample analysis – urine

Urine was analysed by the South Australia Pathology, Automated Laboratory, WCH using the Roche Cobas 501, calibrated as per manufacturer's instructions. Quality control tests were conducted three times per day. Urea was analysed using the kinetic method (Talke & Schubert 1965; Tiffany et al. 1972). Creatinine was analysed using the enzymatic colormetric method. Albumin was analysed using the immunoturbidimetric method (Hubbuch 1991).

3.14 Statistical analysis

A data analysis plan was written in consultation with statisticians, Tom Sullivan and Dr Jennie Louise of the Data Management and Analytical Services, University of Adelaide (DMAC) (Appendix J). The primary outcomes were as per the originally submitted protocol. The statistical tests are described in detail in the data analysis plan.

Permission to collect and analyse data from withdrawn infants was granted. Sex and gestational age at birth (28-29, 30-32 weeks)were adjusted for. Missing data was not imputed. Outliers were not excluded from the primary analysis. Because of the non-normal distribution associated with preterm infant amino acid measures, a Mann-Whitney test was used for phenylalanine, tyrosine and Fisher's Quotient (Graph Pad Prism 6, California, United States of America).

For all of the continuous outcomes (weight gain, length gain, head circumference gain and lean mass gain), a linear effects model with a random intercept and slope was calculated for each infant to estimate the mean weight gain per day for the trial period. Using the slope, a linear regression model was fitted for each infant. Clustering (multiple births) was accounted for by using a generalised estimating equation with an independent working correlation matrix.

3.15 Data management

Data management was the responsibility of the candidate. Intake data were collected from the medical records. Blood chemistry data was collected from the laboratory serial reports. The remaining data was collected from the Clinical Information Services database. The data were entered into the CRF and 10% of completed case report forms were double-checked against source data. The data was then entered into an Excel database. Six infants (10% of participants) were chosen at random and all data points entered in the excel database were checked against the CRF.

3.16 Trial Management Committee

The conduct and management of the RCT was overseen by the Trial Management Committee. Members of the Trial Management committee included: Miss Jessica Reid (PhD candidate), Dr Carmel Collins (primary supervisor, academic neonatal nurse), Professor Maria Makrides (secondary supervisor, research dietitian), Dr Andrew McPhee (tertiary supervisor, consultant neonatologist, Director state-wide neonatal services South Australia), A/Professor Michael Stark (consultant neonatologist).

3.17 Serious Adverse Event Committee

The Serious Adverse Event committee was chaired by A/Professor Ross Haslam, an independent experienced neonatologist. Further members were co-opted if needed as advised by A/Professor Haslam.

Effectiveness of higher protein fortifier on preterm infant growth

Chapter 4 Effectiveness of higher protein fortifier on preterm infant growth

This chapter describes the participant flow, baseline clinical and demographic characteristics, dietary intake, feeding practices, primary outcome, secondary anthropometric results and secondary clinical outcomes. The trial results have been reported according to the Consolidated Standards of Reporting Trials (CONSORT) statement and the extension relating to pragmatic studies (Zwarenstein et al. 2008).

4.1 Participant flow

Two hundred infants were screened between February 2012 and February 2013 for the Poppet trial. Of these 200 infants, 97 were deemed ineligible. Reasons for ineligibility were; likely down transfer to a rural hospital, insufficient milk supply/planned to formula feed, need of an interpreter or the infant had a congenital abnormality (Figure 4.1).

Of the 103 infants that were eligible for the trial, eight were not approached. Five were not approached for sensitivity reasons after discussion with the consultant on duty and three were not able to be approached in the eligibility window for logistical reasons (Figure 4.1).

Ninety-five infants were approached, 35 declined to take part. Reasons given by the parents were; not wanting to take part in research, not wanting the possibility of twins randomised to separate trial arms, already involved in other research and

felt that was enough, the parent did not return to the nursery after the initial approach by the neonatal research nurse and the infant was transferred to Lyell McEwin before the parent had time to consider the trial (Figure 4.1).

Sixty infants were recruited and randomised. Thirty-one were in the high protein group and 29 in the standard group. Two infants were withdrawn from the trial after randomisation but before the first dose of trial product after parents changed their minds about involvement. The parents gave permission for data to be collected and analysed for these infants. One set of twins and one singleton were withdrawn midway through the trial due to perceived feeding intolerance by the parents. One infant was withdrawn from the trial by the clinical team after she developed necrotising enterocolitis.

4.1.1 Randomisation error

Two infants were randomised into the wrong stratum because of a clerical error on the screening log. The infants were randomised as 32 weeks gestation when they were 28 weeks gestation. The infants received their allocated intervention.

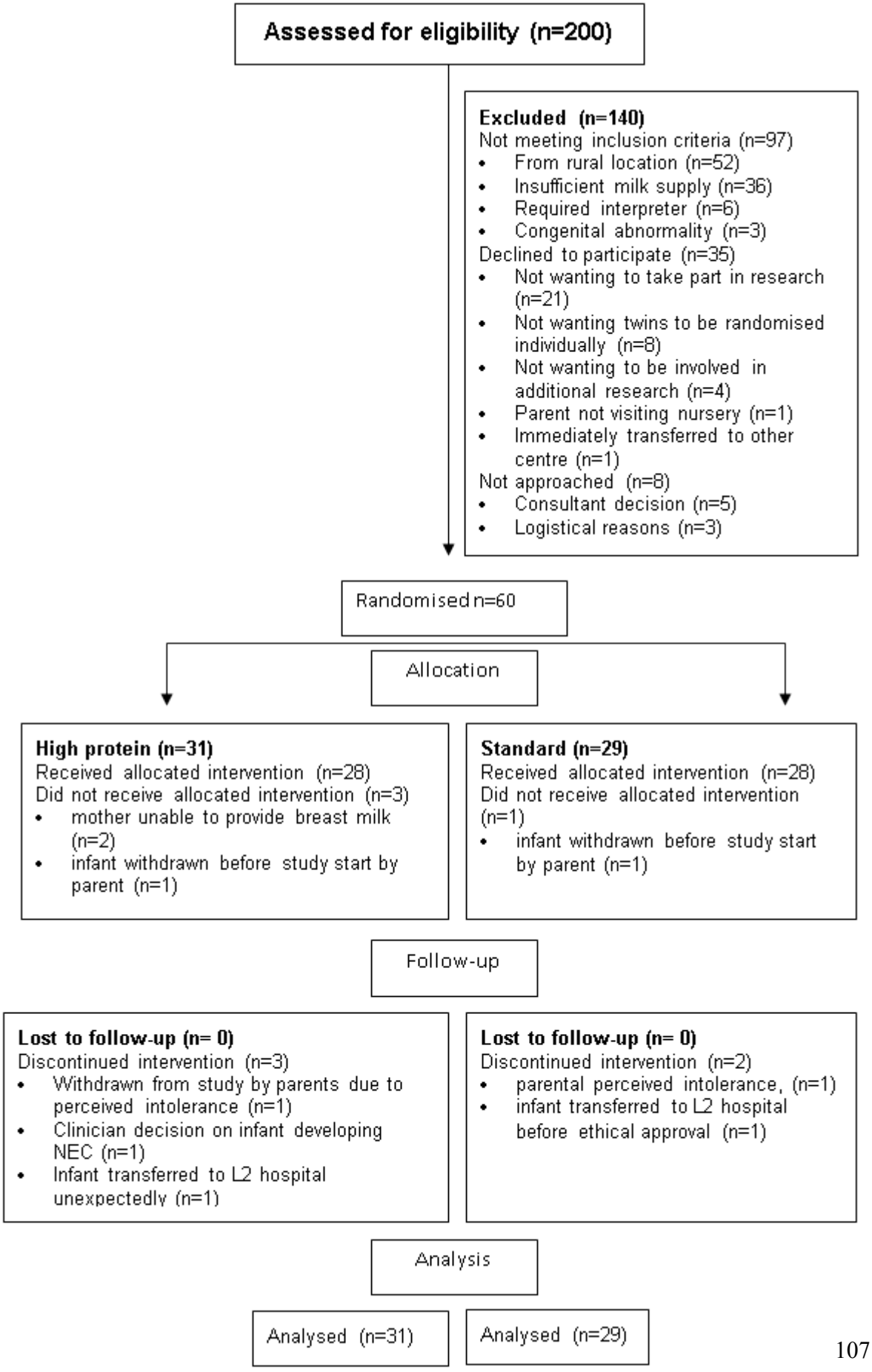


Figure 4.1 Participant flow

4.2 Maternal characteristics at randomisation

General maternal demographics are shown in Table 4.1. Forty five women with 60 infants participated in the trial. There were 31 infants born in multiple births (14 sets of twins, 1 set of triplets). In all multiple births apart from four infants (two sets of twins), the infants were allocated to different interventions. In the case of the triplets, two were randomised to high protein and one to standard protein. The incidence of previous preterm birth was similar between the groups. No mothers had experienced a previous perinatal death. Maternal age, race and incidence of assisted conception were similar between the groups. More mothers in the high protein group left school after year 11, with more mothers completing year 12 in the standard group (Table 4.1). Mothers in the standard group were more likely to have studied after leaving school. Reported smoking and alcohol intake were comparable between the groups (Table 4.1). No adjustments were made on maternal demographics due to the similarity of the groups.

Table 4.1 Maternal demographics*

	High protein n=31, n (%)	Standard n=29, n (%)
Number of mothers	28	28
Maternal age (mean ± SD) years	29.9 ± 6.3	31.7 ± 5.3
Caucasian	27 (87.1)	23 (79.3)
IVF	9 (29.0)	8 (27.6)
Primiparous	19 (61.3)	12 (41.4)
Previous preterm birth•	4 (33.3)	6 (35.3)
Completed any postsecondary education	18 (58.1)	23 (79.3)
Smoking during pregnancy	5 (16.1)	3 (10.3)
Number of cigarettes per week# mean ± sd	5.4 ± 6.0	6.7 ± 8.1
Consumption of alcohol during pregnancy	4 (12.9)	3 (10.3)
Number of alcoholic drinks per week ^ mean±sd	1.4±0.8	1.5±0.9

*Data are reported on the infant level due to individual randomisation of multiple births. •Calculated on mothers that were not primiparous. #Calculated on mothers that answered yes to smoking, High protein n=5, Standard n=3 ^ Calculated on mothers that answered yes to alcoholic consumption, High protein n=4, Standard n=3.

4.3 Birth characteristics at randomisation

The primary reason for preterm birth was similar between the groups with preterm labour and fetal distress being the most common reasons (Table 4.2). Singletons and multiple births were split evenly across the groups (Table 4.3). Antenatal steroid administration was similar between the groups (Table 4.4). There were no differences in Apgar scores between the groups. There were more caesarean births in the high protein group, 58.1% vs 44.8% (Table 4.4).

Table 4.2 Primary reason for preterm birth

	High protein n=31, n (%)	Standard n=29, n (%)
Preterm pre-rupture of membranes	3 (9.7)	1 (3.4)
Spontaneous preterm labour	12 (38.7)	12 (41.4)
Hypertension	2 (6.5)	2 (6.9)
Antepartum haemorrhage	1 (3.2)	4 (13.8)
Suspected intrauterine growth restriction	1 (3.2)	0
Fetal distress	12 (38.7)	9 (31.0)

Table 4.3 Multiple birth demographics

	High protein n=31, n (%)	Standard n=29, n (%)
Singleton	15 (48.4)	16 (55.2)
Twin	15 (45.2)	12 (41.4)
Triplet	2 (6.5)	1 (3.4)

Table 4.4 Administration of steroids and delivery demographics

	High protein n=31 n (%)	Standard n= 29 n (%)
No antenatal steroids	9 (29.0)	7 (24.1)
<24 hours before birth	8 (25.8)	10 (34.5)
Complete dose	11 (35.5)	7 (24.1)
Given >7 days before baby's birth	3 (9.7)	5 (17.2)
Apgar at 1 min mean ± SD	6.9 ± 1.9	7.0 ± 1.9
Apgar at 5 minutes mean ± SD	8.3 ± 1.1	8.5 ± 0.9
Vaginal delivery	13 (41.9)	16 (55.1)
Caesarean section	18 (58.1)	13 (44.8)

4.4 Infant characteristics at randomisation

There were more male infants in the high protein group than the standard group (Table 4.5). The mean completed GA at birth was similar between the groups – high protein 30.5 ± 1.5 (mean ± SD) weeks GA versus standard group 30.1 ± 1.4 weeks GA. Infants randomised to the high protein group were born lighter and shorter than those randomised to standard protein. More high protein infants were born SGA for weight and head circumference at birth. More standard infants were born SGA for length.

Table 4.5 Birth demographics

	High protein n=31, n (%)	Standard n=29, n (%)
Male	16 (51.6)	12 (41.4)
Mean gestational age at birth (weeks) mean ± SD	30.5 ± 1.5	30.1 ± 1.4
28–29 weeks GA	10 (32.3)	9 (31.0)
30-32 weeks GA	21 (67.7)	20 (69.0)
Singleton n	15 (48.4)	16 (55.2)
Birth weight g mean ± SD	1483 ± 423	1551 ± 407
Birth length cm mean ± SD	40.0± 3.3	40.2 ± 2.8
Head circumference cm mean ± SD	28.5 ± 3.0	28.5 ± 1.8
SGA for weight at birth	5 (16.1)	1 (3.4)
SGA for length at birth n	3 (9.7)	5 (17.2)
SGA for head circumference at birth	4 (12.9)	2 (6.9)

4.5 Trial entry

Infants in both groups were randomised seven days after birth with administration of the trial product commencing two days later (Table 4.6). The majority (n=40) of infants received some standard practice HMF (S26 Low Birth Weight Preterm Infant Fortifier, Pfitzer), as they were clinically ready to begin fortifier but their parents had not yet consented to trial participation. Infants in the high protein group received 1.3 ± 1.7 days of standard practice fortifier compared with the standard infants that received 2.0 ± 1.5 days of standard practice fortifier. Thirteen

infants (44.8%) in the high protein group and 7 (25.9%) in the standard group had consented to the trial before standard practice HMF began, so were able to start on whichever Poppet trial arm they had been randomised to without having any standard practice HMF. High protein infants were slightly lighter and shorter than standard infants at trial entry.

Table 4.6 Trial entry summary

	High protein n=31, mean ± SD	Standard n=29 mean ± SD
Days from birth to randomisation	6.6 ± 3.0	7.2 ± 3.0
Days from randomisation to Poppet HMF start	2.0 ± 1.8	1.7 ± 1.6
Infants who only received Poppet HMF n (%)	13 (44.8)	7 (25.9)
Days of standard fortification before trial start	1.3 ± 1.7	2.0 ± 1.5
Days between birth and Poppet HMF start	8.9 ± 3.2	9.0 ± 2.5
Weight at randomisation g	1405 ± 375	1472 ± 314
Length at randomisation cm	40.5 ± 3.1	41.0 ± 2.3
Head circumference at randomisation cm	28.4 ± 2.1	28.5 ± 1.6

4.6 Dietary intake in the first 28 days

High protein infants received more total grams of protein, 7.5 ± 2.7 versus 6.3 ± 2.0 g, and more protein as a proportion of body weight, than standard infants, 4.3 versus 3.4 g/kg/day (Table 4.7). Fluid intake (ml/kg/day) was comparable between the groups suggesting that additional protein did not down-regulate appetite in the High protein group. Intake from direct breast feeds was not included in the total intake.

Table 4.7 Daily intake for the first 28 days of trial

	High protein mean ± SD	Standard mean ± SD
Body weight g	1753 ± 447	1843 ± 452
Recorded milk intake ml	208 ± 100	245 ± 92
Protein from EBM g	2.9 ± 1.5	3.4 ± 1.4
Breast feeds per day	0.5 ± 0.9	0.7 ± 1.0
Poppet fortifier intake ml	15.3 ± 9	19.2 ± 10
Protein from Poppet g	3.4 ± 2.2	2.3 ± 1.3
Formula intake ml*	54 ± 107	26 ± 65
Protein from formula g	1.2 ± 2.4	0.6 ± 1.4
Total protein g	7.5 ± 2.7	6.3 ± 2.0
Total feed volume ml	261 ± 88	271 ± 73
EBM (% of total intake)	83 ± 32	90 ± 23
Protein intake g/kg/d	4.3 ± 1.3	3.4 ± 0.9
Total feed intake m/kg/d	148 ± 34	148 ± 30

*Formula S26 Low Birth Weight Preterm Infant Formula. Protein content 2.2 g/100 ml

4.7 Primary outcome – rate of weight gain

Infants in the high protein group grew at a slower rate than infants in the standard protein group however the difference was not statistically significant in either the unadjusted or adjusted analyses (Table 4.8, Figure 4.2). Data were adjusted for sex and gestational age.

Table 4.8 Primary outcome – mean weight gain per week

			Unadjusted		Adjusted[a]	
	High protein n=31	Standard n=29	Difference	p	Difference	p
Weight gain (g/week)	245 (230, 260)	258 (244,272)	-13 (-32, 5)	0.16	-14 (-32, 4)	0.12

Mean; p represents p value ; 95% CI; [a] Adjusted for sex and gestational age

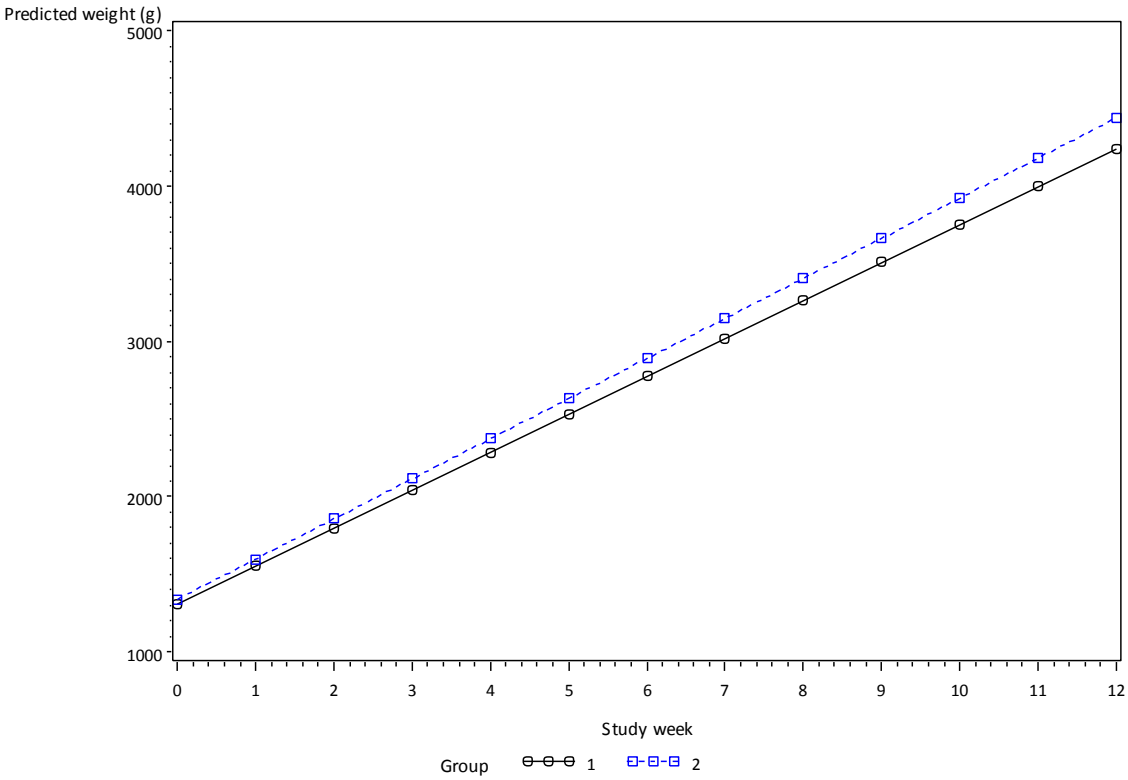


Figure 4.2 Predicted mean weekly weights by group (Group 1, black circles = High protein, Group 2, blue squares = Standard; ITT, adjusted for sex and GA)

4.8 Key secondary outcomes

4.8.1 Length gain

There were no differences in the rate of weekly length gain from randomisation to trial end between groups. This was the case for both the unadjusted and adjusted analyses (Table 4.9, Figure 4.3).

4.8.2 Head circumference gain

There were no differences in head circumference gain per week between the groups. There was also no difference when the data were adjusted for GA and sex (Table 4.9, Figure 4.4).

Table 4.9 Key secondary outcomes – mean length and head circumference gain per week

			Unadjusted		Adjusted[a]	
	High protein n=31	Standard n=29	Difference	p	Difference	p
Length gain (cm/week)	1.1 (1.1, 1.2)	1.1 (1.1, 1.2)	0.0 (-0.1, 0.0)	0.51	0.0 (-0.1, 0.0)	0.45
Head circumference gain (cm/week)	1.1 (1.0,1.1)	1.1 (1.0,1.1)	0.0 (-0.1,0.1)	0.97	0.0 (-0.0, 0.1)	0.79

Mean; p represents p value; 95% CI; [a] Adjusted for sex and gestational age

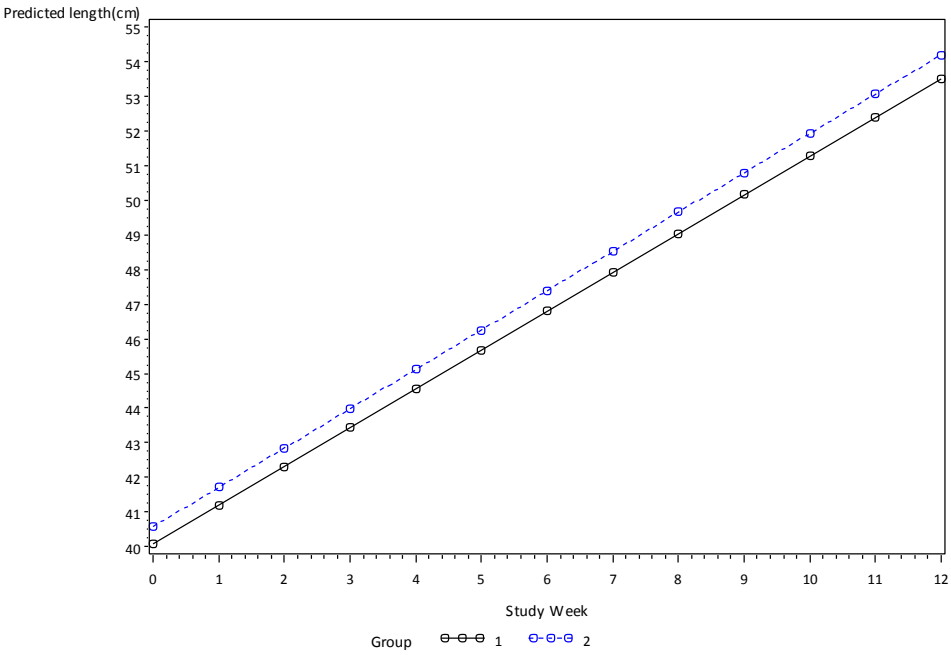


Figure 4.3 Predicted mean weekly lengths by group (Group 1, black circles = High protein, Group 2, blue squares = Standard; ITT, adjusted for sex and GA).

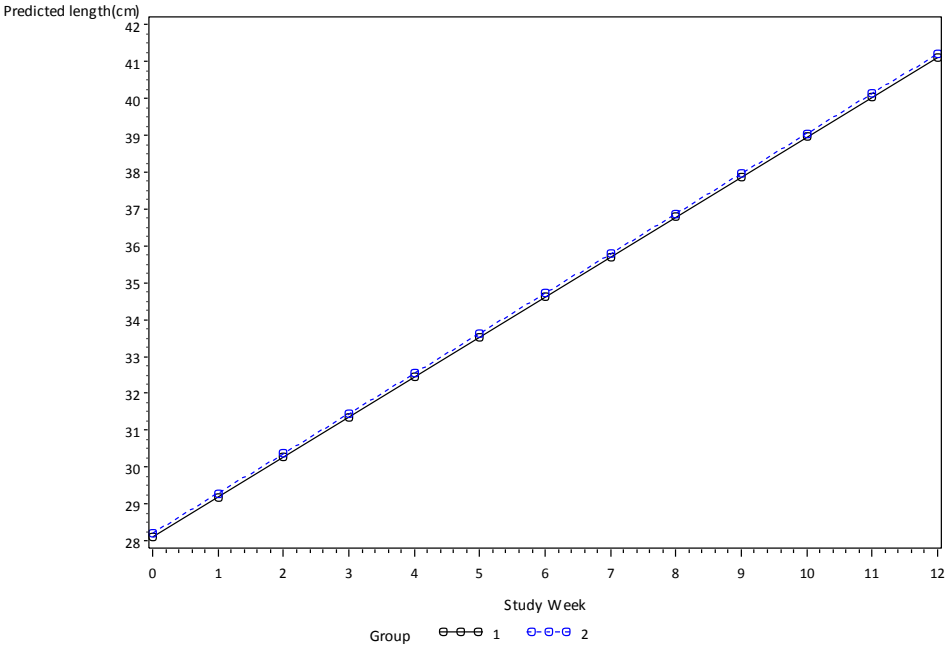


Figure 4.4 Predicted mean weekly head circumferences by group (Group 1, black circles = High protein, Group 2, blue squares = Standard; ITT, adjusted for sex and GA).

4.8.3 Per protocol analyses

To be considered for per protocol analysis infants must have consumed ≥70% of their prescribed Poppet trial fortifier. Forty four (high protein n=21, standard n=23) infants met the criteria. Results from the per protocol analysis did not deviate from those of the intention to treat analysis. There were no differences in growth rates for weight, length or head circumference gain (Table 4.10). The remaining per protocol analyses are included in Appendix K.

Table 4.10 Weekly weight, length and head circumference gains – per protocol

			Unadjusted		Adjusted[a]	
	High Protein n=21	Standard n=23	Difference	p	Difference	p
Weight gain (g/week)	245 (228, 262)	262 (247,277)	-17 (-37, 4)	0.11	-15 (-36, 5)	0.14
Length gain (cm/week)	1.1 (1.1, 1.2)	1.2 (1.1, 1.2)	0.0 (-0.1, 0.0)	0.71	0.0 (-0.1, 0.0)	0.45
Head circ. gain (cm/week)	1.1 (1.1, 1.1)	1.1 (1.1, 1.1)	0.0 (-0.1 , 0.0)	0.67	0.0 (-0.0, 0.1)	0.79

Mean; p represents p value; 95% CI; [a] Adjusted for sex and gestational age

4.8.4 Small for gestation at trial end

There was no difference in the risk of SGA for weight at trial end between the groups (RR 2.5, 95% CI: 0.8, 7.9, p=0.11) (Table 4.11). Risk tended to be increased in the high protein group, albeit not significantly, which is consistent with the imbalance at birth where more high protein infants were SGA. There was no difference in the risk of SGA for length at trial end (RR 1.2 95% CI: 0.8, 1.9, p=0.37) (Table 4.11), though there was a trend for increased risk in the high protein group. Similar results were observed for the measures of SGA for head circumference (RR 2.2, 95% CI: 0.8, 6.3, p=0.10) at trial end, where there was no difference between the groups, but an increased risk in the high protein group –

again likely reflecting the higher numbers of SGA infants in the high protein group at birth.

Table 4.11 SGA at trial end

			Unadjusted		Adjusted[a]	
	High protein n=31 n (%)	Standard n=29 n (%)	RR (95%CI)	p value	RR (95%CI)	p value
SGA for weight	8 (25.8)	3 (10.3)	2.5 (0.8, 7.8)	0.12	2.5 (0.8, 7.9)	0.11
SGA for length	18 (58.1)	14 (48.3)	1.2 (0.8, 1.9)	0.44	1.2 (0.8, 1.9)	0.37
SGA for head circ.	7 (22.6)	3 (10.3)	2.2 (0.8, 6.3)	0.15	2.2 (0.8, 5.9)	0.10

[a] Adjusted for sex and gestational age; p represents p value

4.8.5 Fat free mass as a proportion of body weight

Over the first four weeks of the trial, when >75% of participants were still in hospital, fat free mass as a proportion of body weight was increased in high protein infants from weeks one to four (p=0.03) but with no significant group by time interaction effect (p=0.84) (Figure 4.5, Table 4.12). A post hoc exploratory analysis showed a difference in week three of the trial, such that fat free mass as a proportion of body weight was significantly increased in the high protein group (p=0.04).

Table 4.12 Fat free mass as a proportion of body weight

			Unadjusted		Adjusted[a]	
	High protein	Standard	Difference	p	Difference	p
Fat free mass/body weight Week 1 (n=59)	85 (80, 90)	82 (77, 87)	3 (-3, 10)	0.3	3 (-3, 10)	0.3
Fat free mass/body weight Week 2 (n=57)	93 (88, 97)	87 (82,92)	6 (0, 12)	0.1	6 (0, 12)	0.1
Fat free mass/body weight Week 3 (n=53)	92 (88, 96)	87 (82, 91)	5 (0, 11)	0.04	6 (0, 11)	0.04
Fat free mass/body weight Week 4 (n=46)	92 (86, 98)	90 (84, 96)	2 (-5, 10)	0.5	3 (-5, 11)	0.5

Mean g; 95% CI; p represents p value; [a] Adjusted for sex and gestational age

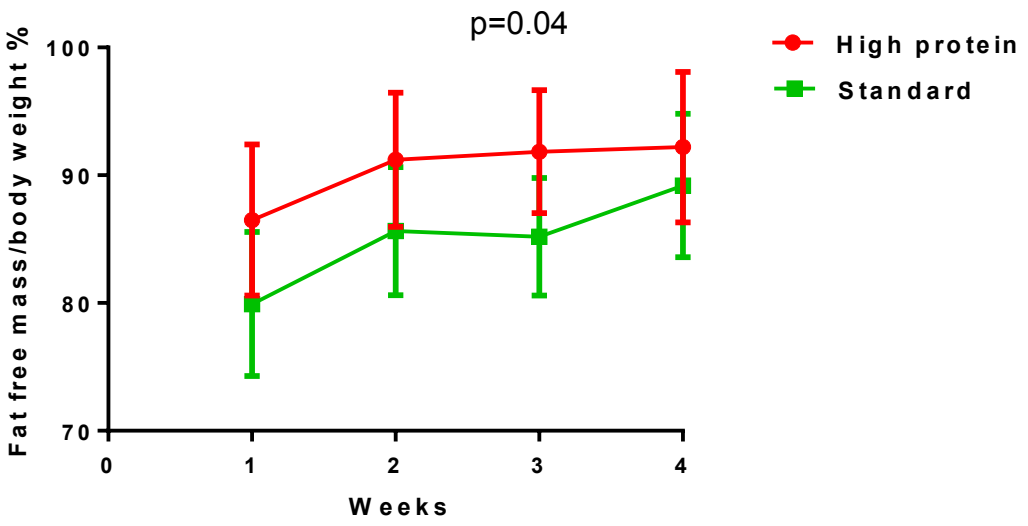


Figure 4.5 Fat free mass as a proportion of body weight for the first 4 weeks of the trial. Adjusted p values. Group p=0.03, Time p=0.01, Group*time=0.84. n=number of infants in the trial at each time point. Week 1 High protein n=30 Standard n=29, Week 2 High protein n=30 Standard n=27, Week 3 High protein n=26, Standard n=26, Week 4 High protein 23, Standard n=23.

4.8.6 Weight, length and head circumference at trial end

Although high protein infants weighed less at trial end this was not significanct in either the unadjusted or adjusted analyses (Table 4.13). This finding is consistent with the difference in birth weight between the groups. There was no difference for length or head circumference at trial end between the groups.

Table 4.13 Anthropometric growth measures at trial end

			Unadjusted		Adjusted[a]	
	High protein n=31	Standard n=29	Difference	p	Difference	p
Weight (g)	2658 (2544,2771)	2757 (2632,2883)	-100 (-254, 55)	0.21	-100 (-250,50)	0.19
Length (cm)	45.2 (44.5, 45.9)	45.8 (45.0, 47.0)	-0.6 (-1.4, 0.3)	0.21	-0.5 (-1.3, 0.3)	0.19
Head circ (cm)	33.1 (32.5, 33.6)	33.0 (32.4, 33.7)	0.0 (-0.6, 0.7)	0.95	0.0 (-0.6, 0.6)	0.92

Mean; p represents p value; CI 95%; [a] Adjusted for sex and gestational age.

4.9 Discussion

This trial was a pragmatic randomised clinical trial designed to test the effect of increasing the protein content of HMF on the rate of weight gain.

4.9.1 Was the intervention successful?

The administration of the intervention ensured that high protein infants received more protein than infants in the standard group. High protein infants received 4.3 g/kg/d protein compared with 3.4 g/kg/d protein for the first 28 days of the trial (Table 4.7). This intake is in line with current international guidelines which recommend a protein intake of 3.5–4.0 g/kg/d for infants 1000–1800 g and 4.0–4.5 g/kg/d for infants <1000 g (Agostoni et al. 2010).

4.9.2 Did the intervention increase the rate of weight gain?

The high protein intervention did not increase the rate of weight gain, the primary outcome of the trial. This result does not support the hypothesis that increased protein fortification of breast milk, when energy content is held constant, will result in an increased rate of weight gain.

Infants in the high protein group were born smaller and remained smaller at trial end. However as the primary outcome was weight gain, not weight at trial end, this is unlikely to have affected the result. This difference was not adjusted for during the statistical analyses as perceived differences at baseline are due to the nature of randomisation.

4.9.3 Did the differences in weight and length at birth and trial start impact on the primary outcome?

There were slight differences between the groups for greater mean birth weight and birth length in the standard group(Table 4.5), which were also observed at trial start (Table 4.6). Due to the strength of the randomisation design, which included stratification by sex and GA, and variable block sizes of four and six, it can be assumed this difference is due to chance and does not suggest that the groups are unbalanced.

Weight gain, the primary and trial-powered outcome, should not be affected by any difference in birth weight. As such, the differences in weight and length at birth and trial start were not adjusted for throughout the analysis. It is unlikely that they affected the final outcome.

4.9.4 Comparison with similar published trials

A comprehensive review of previous studies is included in the literature review chapter of this thesis. However, during the course of this trial, a randomised controlled trial using the same protein content HMF was published (Moya et al. 2012). The multi-centre trial compared a liquid high protein fortifier (3.2 g protein, 81 kcal energy, 4.8 g fat/100 ml EBM) to the standard powder protein fortifier (2.6 g protein, 78 kcal energy, 4.4 g fat/100ml EBM) in an unblinded RCT. Infants were eligible if they were born ≤31 weeks GA and ≤1250g, and were exclusively fed breast milk. One hundred and fifty infants were randomised to either high (n=75) or standard (n=75) protein fortification when enteral intake reached 80 ml/kg/day. The trial period lasted 28 days, or until hospital discharge or termination of fortified breast milk feedings, whichever occurred first.

The primary outcome of the trial was weight gain (g/day). The trial was powered to detect a difference of 1.6 ± 2.8 g/kg/day using a two-tailed test and a power of 80% (p=0.05) in 50 infants. There was a large loss to follow-up with a total of 44 (29%) infants withdrawn from the trial (intervention n=24 32%, control n=20 27%). Eight infants were withdrawn due to fortifier related reasons, 32 infants for reasons not relating to the fortifier and four infants never consumed the fortifier. In the primary analysis, which included all infants that received HMF but not all infants that were randomised (high protein n=51, control n=58), there were no differences in the primary outcome of weight gain. Length gain was found to be greater in high protein infants (0.16 ± 0.006 cm/day versus 0.14 ± 0.006 cm/day, p=0.03). Infants in the high protein group were also found to be heavier and longer at trial day 28.

In comparison, the Poppet trial recruited 60 infants, a smaller number than Moya (2012), however the complete data for all enrolled infants was used for analysis. All clinicians, care givers, participants, researchers and statisticians were blinded to intervention allocation for the duration of the trial, including data analysis. The reasons given for participant withdrawal are clearly described in this thesis. Ten infants (17%) did not complete the trial (two withdrawn by parents before trial start, three withdrawn during trial due to parental perceived intolerance, two were transferred to a rural hospital, one was withdrawn with NEC, two mothers were unable to provide EBM).

The results of the Poppet trial reflect those of Miller et al. (2012) and Tan and Cooke (2008) that have been reviewed at length in the literature review section of this thesis. These studies failed to show a difference in growth rates despite increasing the protein content of fortifier. Miller and colleagues (2012) were unable to improve growth rates when 1.4 g protein/100 ml EBM was compared to 1.0 g protein/100 ml EBM which resulted in median protein intakes of 4.2 g/kg/d versus 3.6 g/kg/d for the first four weeks.

Infants in the Tan trial (2008) were born earlier (26 weeks GA versus 30 weeks GA) and smaller (~900 g vs ~1500 g) than the Poppet trial infants. High protein infants received 4 g/kg/d vs 3.3 g/kg/d for the standard group (Tan did not publish raw additional protein values).

This trial was unable to replicate the increase in growth rates seen by other randomised controlled trials that are reviewed in this thesis.

4.9.5 Delivery system

The intervention product was administered separately to EBM in this study, in order to ensure that as the baby matured and was able to take more feeds directly from the breast, the infant still received fortification. It was not our intention to test the efficacy of the new delivery system as this would have required two additional groups, with high or standard protein fortifier added to EBM as per current practice. The time taken to recruit the required number of infants would have exceeded the PhD timeframe. While not directly testing the delivery system, high compliance of the care staff in administering the trial product and anecdotal evidence from care staff supported the feasibility of the approach. Direct breast feeds for all trial infants were able to be fortified with this method and provide more nutrients than they would have received under current clinical practice. We did not measure direct breast feeds so it was impossible to know whether the increased protein affected the satiety levels of the infants in that group and reduced the amount of milk consumed in direct breast feeds.

4.9.6 Strengths

This trial provides robust evidence due to the strength of the trial design. The recruitment process was transparent and is clearly described in this thesis. Randomisation was performed over the telephone with research staff that otherwise had no involvement in the trial. By choosing to have allocation colours, it was difficult for care staff to ‘guess’ to which colours were allocated to the intervention. The blind for the trial was not broken until after the Steering

Committee reviewed and discussed the results. There was a 100% follow up rate for growth outcomes, with every randomised infant included in the primary analysis. This is the first nutritional intervention trial to include serial body composition measures using Bioelectrical Impedance Spectroscopy. While it is a secondary finding and to be interpreted with caution, there is evidence to suggest that infants in the high protein group preferentially accrued more lean mass as a proportion of body weight than infants in the standard group.

Biochemistry, feeding, respiratory and clinical outcomes

Chapter 5 Biochemistry, feeding, respiratory and clinical outcomes

Safety outcomes for the Poppet trial were specified in the Protocol (Appendix A) and included blood chemistry markers, BUN, albumin, creatinine, glucose, base excess and pH. Feeding safety outcomes included incidence of NEC, feeding intolerance and need for feeds to be supplemented. Other clinical data is also reported in this chapter.

5.1 Blood chemistry safety outcomes

Blood samples were collected weekly from randomisation. Due to the variable nature of blood chemistry data and length of hospital stay (to discharge), only the first three trial weeks were able to be accurately analysed using a linear mixed effects model. This is also the time when the infant is the most vulnerable and protein intake increases rapidly. Graphs in this chapter are plotted with unadjusted data points, unadjusted 95% confidence interval values and adjusted p values when significance occurred.

5.1.1 BUN measurements from randomisation to week 3

There was a significant group by time interaction for the blood urea nitrogen levels ($p<0.001$) with the blood urea nitrogen levels significantly increased in the high protein group (Figure 5.1, Table 5.1). This difference was maintained over the duration of the intervention ($p=<0.001$).

There were 12 occurrences in nine separate infants where BUN levels were measured as over the pre-specified safety threshold of 8 mmol/L.

Seven occurrences of BUN exceeding 8 mmol/L occurred during baseline blood tests taken at randomisation, so were not a result of the intervention. All of these occurred without a base excess measurement of ≤-6 mmol/L. Six of these seven infants recorded BUN levels in the normal range at their next weekly blood test. One infant recorded a BUN measurement ≥8 mmol/L at week one with base excess of >-6 mmol/L. The uraemia resolved spontaneously and the infant did not show another BUN measurement of over 8mmol/L for the course of the trial. One infant that had recorded a normal baseline BUN measurement showed a BUN ≥8 mmol/L at the week one blood test. As this was not accompanied by a base excess ≤-6 mmol/L the attending consultant was satisfied for the infant to remain on the trial. The uraemia resolved spontaneously by the next weekly blood test. One infant experienced a 12 day period of BUN measurements ≥8 mmol/L, peaking at 8.8 mmol/L. Because base excess did not reach ≤-6mmol/L and as there were no other abnormal biochemical blood markers detected the infant was allowed to remain in the trial under careful consultant observation. The five instances that occurred in the trial period were all in the high protein group. At no time point did any infants in the trial have a base excess reading of <-6 mmol/L.

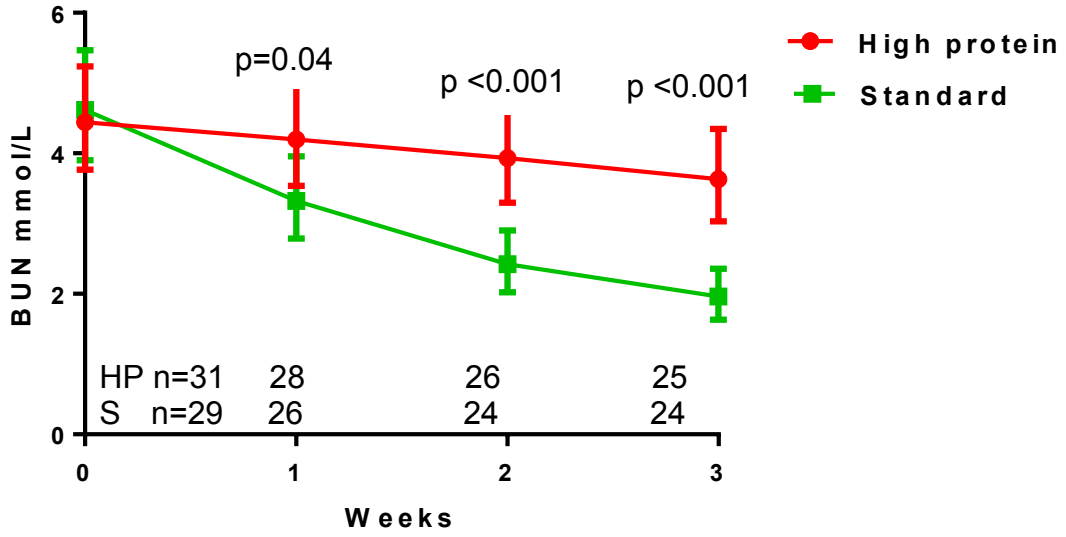


Figure 5.1 BUN from randomisation to week 3. Overall Group <0.001, weeks <0.001, group*weeks <0.001.

Table 5.1 BUN values from randomisation to week 3

			Unadjusted		Adjusted[a]	
BUN mmol/L	High protein	Standard	Ratio	p	Ratio	P
Week 1	4.4 (3.8, 5.2)	4.6 (3.9, 5.5)	1.0 (0.8, 1.2)	0.73	1.0 (0.8, 1.2)	0.78
Week 2	4.2 (3.5, 5.0)	3.3 (2.8, 3.9)	1.3 (1.0, 1.6)	0.05	1.3 (1.0, 1.6)	0.04
Week 3	3.9 (3.3, 4.3)	2.4 (2.0, 2.9)	1.6 (1.3, 2.1)	<0.001	1.6 (1.3, 2.1)	<0.001
Week 4	3.6 (3.0, 4.3)	2.0 (1.6, 2.4)	1.9 (1.5, 2.4)	<0.001	1.9 (1.5, 2.4)	<0.001

Mean; 95% CI; p represents p value, [a] Adjusted for sex and gestational age

5.1.2 Base excess measurements from randomisation to week 3

There was a group by time interaction for base excess effect, with high protein infants recording lower measurements (p=0.034) (Figure 5.2). At week 2, there was a difference between the groups, with the high protein group showing a lower base excess (p=0.04).

5.1.3 Albumin measurements from randomisation to week 3

There was no group by time interaction effect or group difference for albumin levels (adjusted interaction group*weeks p=0.27) (Figure 5.3).

5.1.4 Creatinine from randomisation to week 3

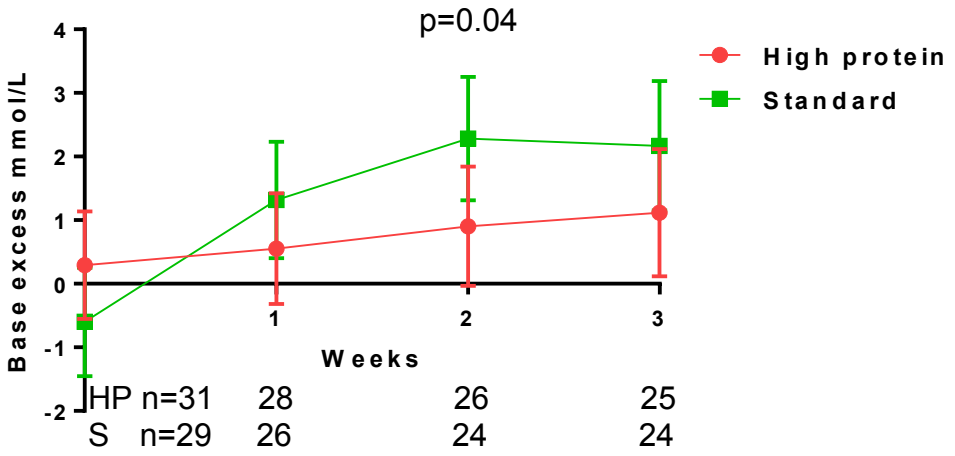
There was no group by time interaction effect or group difference for creatinine levels (Figure 5.4).

5.1.5 Glucose from randomisation to week 3

There was no group by time interaction or group difference for glucose effect (Figure 5.5).

5.1.6 pH from randomisation to week 3

There was no group by time interaction or group difference for pH effect (Figure 5.6).



Adjusted interaction group p=0.169, weeks p<0.001 group*weeks p= 0.034

Figure 5.2 Base excess from randomisation to week 3

Table 5.2 Base excess measurements from randomisation to week 3

			Unadjusted		Adjusted	
Week	High protein	Standard	Difference	p	Difference	p
0	0.3 (-0.6, 1.1)	-0.6 (-1.5, 0.3)	0.9 (-0.3, 2.0)	0.13	0.9 (-0.3, 2.1)	0.13
1	0.6 (-0.3, 1.4)	1.3 (0.4, 2.2)	-0.8 (-2.0, 0.5)	0.22	-0.7 (-2.0, 0.5)	0.24
2	0.9 (-0.0, 1.8)	2.3 (1.3, 3.3)	-1.4 (-2.7, -0.1)	0.04	-1.4 (-2.7, -0.0)	0.04
3	0.1 (0.1, 2.1)	2.2 (1.2, 3.2)	-1.1 (-2.4, 0.3)	0.14	-1.0 (-2.5, 0.4)	0.14

Mean; p represents p value; CI:95%, [a] Adjusted for sex and gestational age.

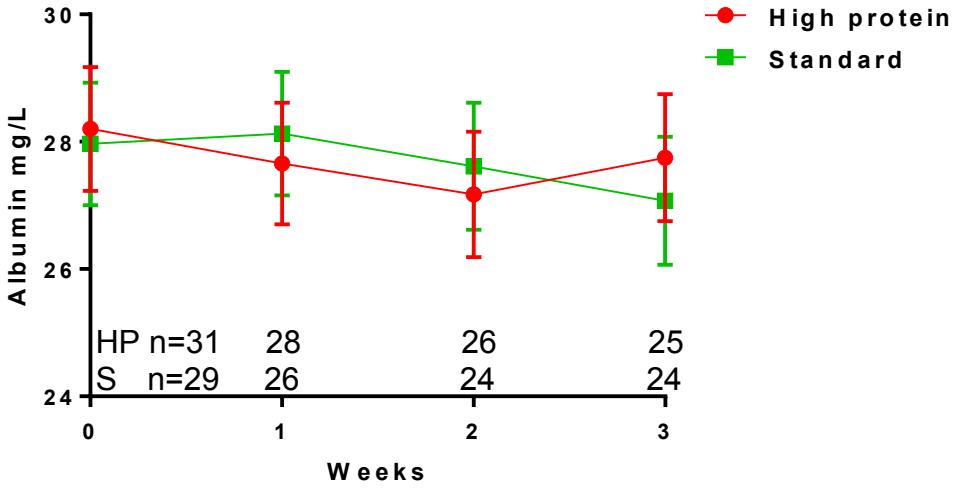


Figure 5.3 Albumin from randomisation to week 3

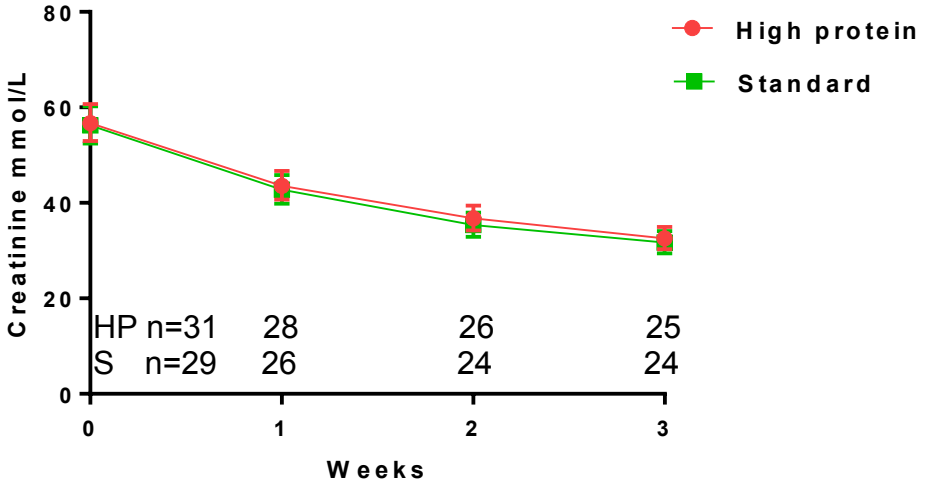


Figure 5.4 Creatinine from randomisation to week 3

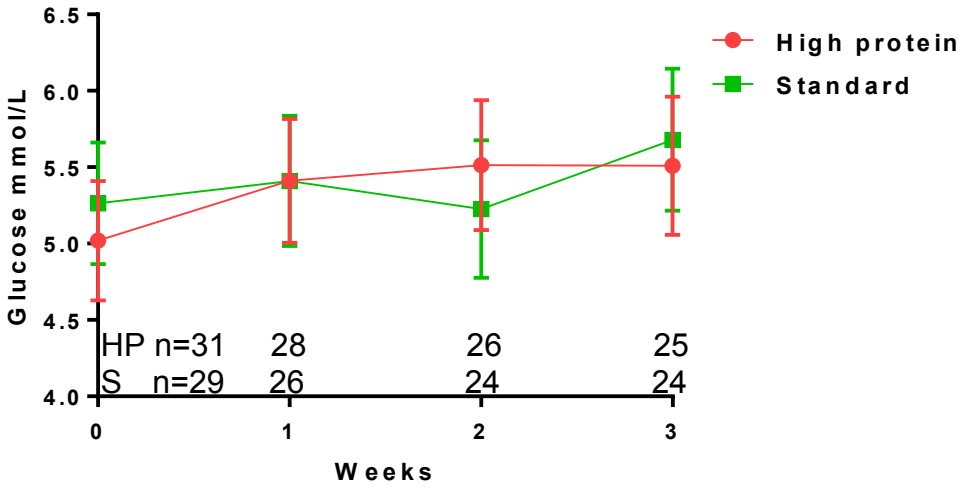


Figure 5.5 Glucose from randomisation to week 3

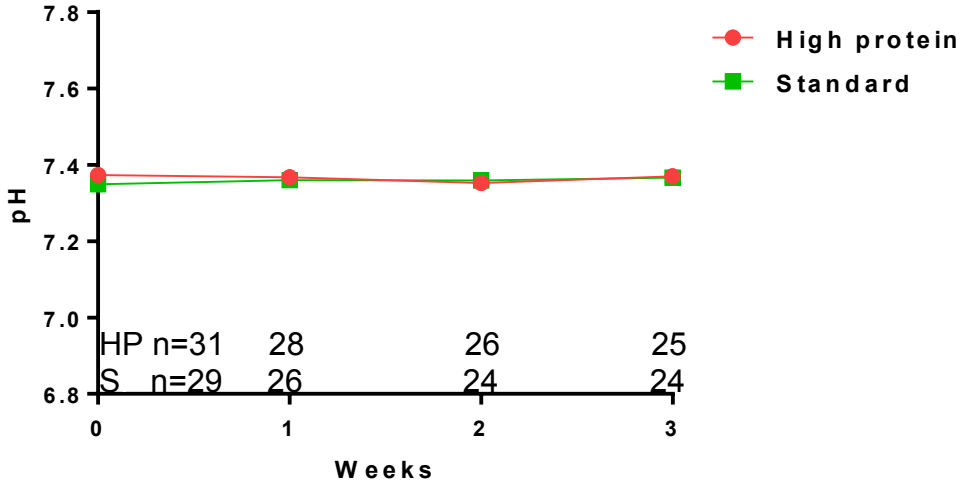


Figure 5.6 pH from randomisation to week 3

5.1.7 Amino acid measures

Phenylalanine and tyrosine, amino acids associated with increased protein intake and toxicity, were both increased in the high protein group compared to standard. Fisher's Quotient, a measure of liver stress, did not differ between the groups. The range of phenylalanine was similar between the groups.

Table 5.3 Blood amino acid measures at week 3

			Unadjusted	
	High protein median (range) n=28	Standard median (range) n=26	Difference	p value
Phenylalanine μmol/L	32.96 (22-59)	24.46 (17-58)	8.5	<0.001
Tyrosine μmol/L	196.0	128.0	68.1	<0.003
Fisher's Quotient^	1.17	1.29	0.12	0.23

^Fisher's Quotient expressed as a molar ratio (leucine, valine, isoleucine/ phenylalanine, tyrosine)

5.2 Urine chemistry outcomes

Urine samples were collected every two weeks from randomisation. Due to the difficulty of collecting a clean, non-invasive sample from a preterm infant without a catheter, and the differing lengths of stays between the infants, a pragmatic statistical approach was taken for these data. When no interaction (group by time)

difference was found, data from all time points were combined to detect an overall difference between the groups using estimated geometric means

5.2.1 Urinary urea, creatinine and albumin

There was a significant difference in urine urea levels between the groups when data from all time points were combined. Urine urea was higher in the high protein infants when compared to the standard infants (<0.001). There were no differences between the groups for creatinine or albumin.

Table 5.4 Urine Urea, creatinine and albumin

			Unadjusted		Adjusted[a]	
	High protein n=62	Standard n=46	Ratio	p	Ratio	p
Urea mmol/L	53 (47, 60)	35 (30, 40)	1.5 (1.3, 1.8)	<0.001	1.5 (1.3, 1.9)	<0.001
Creatinine mmol/L	0.85 (0.72, 0.99)	0.84 (0.71, 1.00)	1.01 (0.82, 1.24)	0.95	0.97 (0.76, 1.29)	0.82
Albumin Mg/L	17 (13, 22)	17 (13, 22)	1.0 (0.7, 1.5)	0.96	0.9 (0.6, 1.4)	0.66

Mean; p represents p value; 95% CI; [a]Adjusted for sex and gestational age.

5.3 Feeding outcomes

5.3.1 Infants that had feeds withheld

If a feed (or feeds) were not given, that day was recorded as feeds withheld. More infants in the high protein group had feeds held with 11 (35%) versus 6 (21%) infants having feeds withheld for at least part of one day. High protein infants had a significantly higher likelihood of having feeds withheld (RR 3.12, CI 95%: 1.3, 8.0, p=0.01).

5.3.2 Feeding tolerance

Infants in the high protein group spent more days on parenteral feeds (10 days versus 9) but this difference was not significant. There were no differences in days of intravenous lipid or time to reach full enteral feeds (150 ml/kg/day) (Table 5.5).

Table 5.5 Feeding tolerance outcome

			Unadjusted		Adjusted[a]	
	High protein n=31	Standard n=29	Ratio	p	Ratio	p
Days of parenteral feeds	10 (7, 13)	9 (7, 11)	1.2 (0.8, 1.6)	0.34	1.8 (0.9, 1.6)	0.28
Days of Lipid feeds	4 (3, 7)	4 (3, 6)	1.1 (0.7, 1.8)	0.72	1.1 (0.7, 1.8)	0.61
Days to full enteral feeds	8 (6, 10)	8 (7, 10)	1.1 (0.8, 1.4)	0.72	1.1 (0.8, 13.7)	0.71

Mean; p represents p value; 95% CI; *Adjusted for sex and gestational age.

5.3.3 Incidence of Necrotising enterocolitis and supplementation

One infant in the high protein group was diagnosed with NEC. One infant in the standard group had feeds supplemented with Protifar (Table 5.6).

Table 5.6 Incidence of Necrotising enterocolitis and supplementation

	High protein n (%), n=31	Standard n (%), n=29	p value
Incidence of NEC	1 (3.2)	0	>0.99^
Feeds supplemented	0	1 (3.4)	0.48^

n (%); ^Fisher's exact

5.4 Respiratory outcomes

High protein infants were more likely to be diagnosed with normal respiratory health, but this was not significant (RR 0.9, 95% CI: 0.65–1.22, p=0.48; adjusted, RR 0.96, 95% CI: 0.64–1.16, p=0.32, data not shown). There was also a non-significant increase in the risk of being discharged home on oxygen in the high protein group (n=2, 6.5%) compared to the standard group (n=1, 3.4%) (RR 1.87, 95% CI: 0.19–18.75, p=0.59,). There were no differences in administration of high frequency oscillation ventilation (HFOV) between the groups (n=2, 6.5% vs n=2, 6.9%) (RR 0.94, 95% CI: 0.09–9.38, p=0.94). The incidence of chronic lung disease was higher in the high protein group (n=3, 9.7% versus n=1, 3.4%) but this difference was not significant (RR 2.81, 95% CI: 0.32–24.61, p=0.35). There was no difference in the need for postnatal steroids (HP n=2 6.5%, S n=2 6.9%, RR 0.94, 95% CI: 0.09–9.38, p=0.94). Due to the low frequency of the secondary clinical outcomes, it was not always possible to perform an adjusted analysis. Similarly, a risk ratio could not always be calculated and a Fisher's Exact Test 2-sided analysis was performed. There were no differences in the requirement for Nitric Oxide between the groups (Table 5.7)

Table 5.7 Need for respiratory support and incidence of chronic lung disease

	High protein n (%), n=31	Standard n (%), n=29	Unadjusted RR mean (95% CI)	p value
Home oxygen	2 (6.5)	1 (3.4)	1.87 (0.19-18.75)	0.59
Need for HFOV	2 (6.5)	2 (6.9)	0.94 (0.15-5.90)	0.94
Nitric oxide	0	1 (3.4)		0.48^
Chronic lung disease	3 (9.7)	1 (3.4)	2.81 (0.32-24.61)	0.35
Postnatal steroids	2 (6.5)	2 (6.9)	0.94 (0.15-5.90)	0.94

^Fisher's Exact Test

5.4.1 Mean hours of ventilator mediated support

There was a non-significant trend for increased dependence on respiratory therapy in the high protein group. The mean hours of intermittent positive pressure ventilation (IPPV) (12.8 ± 35.3 versus 7.5 ± 18.4 hours); constant positive airway pressure (CPAP) (30.8 ± 54.5 versus 25.4 ± 36.2 hrs); humidified high flow nasal cannula therapy (HHFNC) (74.2 ± 180.0 vs 69.2 ± 188.0 hours); and total respiratory support (117.8 ± 219.6 vs 102.1 ± 202.5 hours) were increased in the high protein group (Table 5.8).

Table 5.8 Hours of IPPV, CPAP and HHFNC

			Unadjusted		Adjusted[a]	
	High protein	Standard	Ratio	p	Ratio	
Hours of IPPV	12.8 (4.8, 34.5)	7.5 (3.3, 17.0)	1.7 (0.5,6.1)	0.40	1.8 (0.5, 6.6)	0.35
Hours of CPAP	30.8 (16.5, 57.6)	25.4 (15.2, 42.5)	1.2 (0.5, 2.8)	0.64	1.2 (0.6, 2.6)	0.64
Hours of HHFNC	74.1 (31.7,173.8)	69.2 (25.5, 188.0)	1.1 (0.3, 3.9)	0.92	0.9 (0.3, 2.5)	0.79
Hours of Resp. Support	117.8 (61.2,227.0)	102.1 (47.8,218.0)	1.6 (0.5, 3.0)	0.77	1.0 (0.4, 2.3)	0.97

Mean; p represents p value; 95% CI; Number of infants that received IPPV, High protein n=6, Standard n=8, CPAP, High protein n=19, Standard n=16, HHFNC, High protein n=10, Standard n=10.[a] Adjusted for sex and gestational age

5.5 Other clinical outcomes

The incidence of IVH, cerebral cyst, surgery, NED participation, retinopathy of prematurity and late onset sepsis were secondary outcomes that occurred infrequently. As predicted there were no differences between the groups (Table 5.9).

Table 5.9 Other clinical outcomes

			Unadjusted		Adjusted[a]	
	High protein n=31, n (%)	Standard n=29, n (%)	RR (95% CI)	p	RR (95% CI)	p
Any IVH	3 (9.7)	1 (3.4)	2.9 (0.3, 31.1)	0.39	3.0 (0.3, 25.0)	0.32
Any cerebral cyst	0	1 (3.4)		0.47[#]		
Surgery	3 (9.7)	2 (6.9)	1.40 (0.25, 7.85)	0.70[#]		
NED participation*	9 (29.0)	7 (24.1)	1.20 (0.63, 2.30)	0.58[#]		
Retinopathy of prematurity	0	1 (3.4)		0.43[#]		
Late onset sepsis	1 (3.2)	0		>0.99^[#]		

[#] Unadjusted analyses only due to small n numbers; p represents p value; [a] Adjusted for sex and gestational age. *Neonatal Early Discharge (NED) allows infants to go home with a naso-gastric feeding tube and is offered to families on a case by case basis. Eligibility criteria include but are not limited to, good weight gain, ability of mother to administer gavage feeds and that the family live in close proximity to WCH. The infant is visited by a neonatal nurse as frequently as required, typically daily in the first instance. Mothers in the NED program were taught to administer the trial product and kept a record of compliance and fluid intake.

5.6 Discussion

Significantly elevated BUN levels at weeks 1, 2 and 3 were seen in the high protein infants. This finding was expected and is supported by other high protein nutritional intervention studies that have also shown an increased BUN levels (Arslanoglu, Moro & Ziegler 2006; Miller et al. 2012; Moya et al. 2012). Assuming adequate renal function, BUN is proportional to protein intake (Polberger, Axelsson & Raiha 1990) and therefore often used as a crude marker of protein sufficiency. Low BUN levels suggest inadequate protein intake and high levels indicate possibly excessive intake (Arslanoglu, Moro & Ziegler 2006). In this study base excess was lower in the high protein infants but still within the reference range for preterm infants.

Urine urea was found to be significantly higher in the high protein infants, as anticipated by the high BUN. There were no differences in creatinine, albumin, or other biochemical markers suggesting that the intervention did not cause harm.

Infants in the high protein group were more likely to have had feeds withheld. Calculated as a daily binary outcome, if any feed was withheld then that day was recorded as a withheld. It is possible that the protein content of the intervention contributed to this finding. However, there were no differences between days to reach full enteral feeds, days of parenteral feeds or days of lipid feeds. If the high protein infants were not tolerating the increase in protein, then it is likely that these outcomes would also show differences. As this outcome is secondary, it is unknown whether this finding is directly related to the intervention. There were no differences in respiratory and other clinical outcomes. The slight skew towards

poorer clinical outcomes in the high protein infants is possibly explained by the group showing a smaller weight at birth (Table 4.2) rather than a result of the intervention.

More infants in the high protein group were diagnosed with chronic lung disease (3 versus 1), which is reflected in the increased mean number of hours of respiratory support prescribed to these infants. While it can't be ruled out that the high protein intervention increased the incidence of chronic lung disease, a causal relationship is extremely unlikely given the complex nature of the disease. Similarly, in the high protein group, one infant contract late-onset sepsis and one infant developed NEC with no occurrences in the standard group. Again, although it must be considered, it is unlikely that either incidence was caused by the intervention. What is more certain is the detrimental effect that chronic lung disease, late onset sepsis and NEC have on growth. It is possible that the trend for decreased growth in the high protein group was influenced by a cluster of infants that contracted illnesses which impede growth. The incidence of NEC was immediately reported to the Women's and Children's Hospital Ethics Committee. The infant was discharged home after surgery. The Serious Adverse Events Committee could not rule out the intervention contributing to the NEC but deemed the intervention 'unlikely' to have caused the NEC. While the cause of NEC is unknown, the incidence is not increased when infants are fed fortified milk (Kuschel & Harding 2004). High osmolarity substances, such as medications, have been suggested as possible causes but causation has not been shown (Lampkin et al. 2013). The study solution in the Poppet trial was immediately diluted by the milk feed which followed

and did not exceed the current ESPGHAN osmolarity recommendation of 490 mmol/L which is within the range of other published trials (McLeod 2010).

The higher BUN, urinary urea and slightly higher (more negative) Base Excess values in the high protein group reflect the higher protein load, assuming no difference in renal function between the groups. However, the values seen were not of clinical significance. Median phenylalanine and tyrosine levels at 3 weeks of age were slightly higher in the high protein group, presuming reflecting the use of Protifar (a bovine milk protein product); note however that the ranges of these amino acids were similar and within the normal range for preterm infants.

There was no difference in Fisher's Quotient between groups. This is not routinely measured in preterm infants due to the transient rises and falls in amino acids levels they experience. It has been included in this thesis as a pragmatic tool for estimating hepatic stress that may have been induced by the increased protein (Soeters & Fischer 1976).

General discussion

Chapter 6 General discussion

Immature organs, coupled with high metabolic needs, makes adequate nutrition of the preterm infant a clinical challenge. The advantages of breast milk as a primary food source have been well documented, both in this thesis and in the literature. However, breast milk alone does not meet all nutritional requirements and breast milk fortification is standard clinical practice. In recent years, a number of randomised controlled trials have attempted to determine the ideal protein fortification combination to achieve the gold standard preterm growth rate, that is, the equivalent growth rate and lean mass accumulation that the infant would experience if still in utero. An additional consideration is the variable breast milk protein content both between and within mothers.

The aims of this thesis were to:

i) Conduct a randomised controlled trial comparing weight gain in breast fed preterm infants fed a high protein content human milk fortifier compared with standard protein content human milk fortifier. In order to address the issue of fortifying direct breast feeds, the fortifier was be given as an entrée before the feed.

ii) Develop preterm infant specific constants to allow body composition to be calculated using Bioelectrical Impedance Spectroscopy.

In order to address the first aim, a randomised controlled trial was conducted recruiting 60 preterm infants (born 28–32 weeks gestation) that had breast milk feeds supplemented with additional protein or the current clinical practice standard

amount of protein. Trial start was from randomisation with trial end occurring when the naso-gastric tube was removed (usually one day before hospital discharge). The weight of the infant was measured daily. Length, head circumference and body composition were measured weekly. Blood chemistry, (including BUN, creatinine, albumin, base excess, pH, glucose and amino acids) was measured weekly. Urine samples were collected every two weeks and analysed for urea, creatinine, albumin and amino acids. All data were analysed as intention to treat with no outliers removed.

To address aim two, a BIS validation trial consisting of 99 preterm infants (born <37 weeks gestation) was conducted at WCH and FMC.

TBW and ECW volumes were determined in a cohort of preterm infants by the reference method of tracer dilution, using deuterium and sodium bromide (NaBr) for TBW and ECW, respectively. BIS measurements were obtained to allow determination of R_∞ and R_0 and thus infant specific constants. The constants were assessed in a separate group of infants using these coefficients to predict TBW and ECW from BIS measurements and to compare these predicted values with values obtained using the reference methods.

6.1 Key findings

The trial was unable to achieve the primary outcome of increasing weight gain per week by increasing protein fortification to 1.8 g/100 ml EBM (compared with standard fortification of 1.0 g/100 ml EBM). Increasing protein fortification

increased protein intake and BUN but did not increase weight, length or head circumference gain. Lean mass as a percentage of body weight was increased in the high protein infants, possibly indicating that increased protein results in more lean tissue accruement. However, this is a secondary finding and to be interpreted with caution.

The second part of this PhD involved determining resistivity coefficients for use of determining body composition using BIS. For the first time, body composition can be known in preterm infants using a method that is immediate, inexpensive, non-invasive and can be performed cot-side.

6.2 Strengths

At the time of conception, this trial proposed giving the highest protein content human milk fortifier to preterm infants. With 60 preterm infants enrolled, this randomised controlled trial is one of the largest nutritional trials conducted in the preterm population. Randomisation was stratified for gestational age and sex. Twins were randomised as individual infants to avoid clustering.

Blinding of care givers, researchers, statisticians and participants was maintained at all times. Average enteral protein intake for the first 28 days of the trial in the high protein group reached recommended guidelines. The per protocol analysis included 70% of randomised infants.

By analysing the protein content of EBM, an exact amount of protein received by each infant was known, removing reliance on published averages which might not match our population.

6.3 Limitations

The PhD timeframe limited trial design, with authors ideally including an extra two groups. High protein fortifier mixed directly into EBM and standard fortifier mixed directly into EBM, in order to maintain blinding and fortify direct breast feeds

Absence of measuring body composition at randomisation was a limitation of trial design, although it is unlikely that result would be different if that measure had been collected. As all other collected outcomes were similar at randomisation it could be safely assumed that lean mass was also similar.

6.4 Implications

The results of this clinical trial suggest that the current fortification guidelines are adequate even though they do not meet guideline requirements or provide adequate nutrition to meet the equivalent in utero growth rate. The data presented suggests that the organs of the immature preterm infant are unable to process and utilise increased protein for lean tissue growth. That there was increased urea in the urine of the high protein infants suggests that the preterm infant may already be saturated with protein at the current fortification guidelines. It is possible that

the preterm infant may require increased carbohydrate when additional protein is administered to ensure the protein can be used for lean tissue growth.

The validation of BIS for body composition in preterm infants is a success for the field. The ability to detect changes in body composition using an inexpensive, cot-side device is exciting. While BIS does not provide valid individual results, it gives great insight on a population level and thus is extremely valuable to researchers.

The effect of this intervention of later growth, body composition or functional outcomes is difficult to determine.

6.5 Future directions

Coupled with a similar trial which also added 1.8 g/100 ml EBM, it is the recommendations from this thesis that preterm infants are unable to effectively utilise protein for growth administered at or over 1.4 g/100 ml EBM.

It is possible that increasing carbohydrate content of fortifier may increase growth rate in preterm infants and a trial to test this is recommended. A protein supplement with a greater whey to casein ratio may result in improved growth. It is also possible that the current gold standard for preterm growth, that is, the equivalent in utero growth, is unattainable due to the immaturity of preterm organs and difficulty in digesting food.

Future research should be directed to optimising neonatal growth using an individualised diet, based on breast milk protein content and blood urea nitrogen levels.

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Appendices for the thesis entitled:

The effect of higher protein human milk fortifier on growth in preterm infants

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May 2014

Appendices

Appendix A - Protocol

1. **Title**

Does increasing the protein content of human milk fortifier improve growth in preterm infants <33weeks gestation? A randomised controlled trial.

2. **Investigators and Qualifications.**

Chief Investigator A	**Miss Jessica REID** BSc Hons, PhD candidate **Child Nutrition Research Centre** Women's and Children's Health Research Institute 72 King William Road North Adelaide, SA 5006 jessica.reid@adelaide.edu.au Tel: 8161 6848
Chief Investigator B	**Dr Carmel COLLINS** RN RM NICC BSocSc GDPH PhD Research Fellow Child Nutrition Research Centre Women's and Children's Health Research Institute Flinders Medical Centre Bedford Park SA 5042 carmel.collins@health.sa.gov.au Tel: 8204 5755
Chief Investigator C	**Dr Andrew McPHEE** MBBS, FRACP Paed Director Neonatal Intensive Care Unit Dept Neonatal Medicine Women's and Children's Hospital 72 King William Road North Adelaide, SA 5006 andrew.mcphee@health.sa.gov.au Tel: 8161 7631
Chief Investigator D	**Dr Michael STARK** MBChB, PhD Dept Neonatal Medicine Women's and Children's Hospital 72 King William Road North Adelaide, SA 5006 michael.stark@adelaide.edu.au Tel: 8161 7631

Chief Investigator E	**Prof Maria MAKRIDES** Bsc, BND, PhD Executive Director, Women's and Children's Health Research Institute 72 King William Road North Adelaide, SA 5006 maria.makrides@health.sa.gov.au Tel: 8161 6067

3. **Purpose of Study** (including hypothesis to be tested)

The aim of this study is to compare weight gain in breast fed preterm infants <33weeks fed a high protein content human milk fortifier (1.8 g/100 ml) with infants fed a standard protein content human milk fortifier (1.0 g/100 ml).

We hypothesise that a high protein entree of human milk fortifier administered before each feed will improve weight gain.

4. **Background**

Current nutritional management practices are failing the preterm infant. While cardiac and respiratory health has dramatically improved in the last two decades, nutrition has floundered. Recent evidence suggests that growth in the fetal and neonatal period is important for adult health. Slow neonatal growth is strongly associated with poorer neurodevelopment (Ehrenkranz, Dusick et al. 2006). However care must be taken as there is evidence suggesting an association between rapid neonatal growth and metabolic disease in adult life (Uthaya, Thomas et al. 2005).

<u>Too many infants discharged small for gestational age (SGA)</u>

Different neonatal units report different rates of SGA at discharge. A recent local study of 138 preterm infants born <33 weeks gestation at the Women's and Children's Hospital by Collins and colleagues (Collins, Gibson et al. 2008) reported 8% and 13% were SGA for weight and length respectively at birth. By the time of discharge home, 32% of infants were SGA for weight and 55% for length. The poorest growth was in the subgroup of infants born <28 weeks gestation (n=38). While 6% of infants were SGA for weight at birth, this increased to 34% at discharge. The same decline was evident for length where 3% of infants were SGA for length at birth and 77% were at discharge. Olsen and colleagues (Olsen, Lawson et al. 2009) report similar findings in an analysis of 1214 infants born 26-29 gestational age. At birth, 12% of infants were SGA for weight. This increased to 21% SGA-for weight at discharge. The findings of these studies indicate that current nursery practices are failing to meet the growth requirements of the preterm infants.

<u>Poor in-hospital growth is associated with poor neurodevelopment outcomes</u>

The consequences of poor neonatal growth in preterm infants are well documented being associated with poor health outcomes in the immediate and long term (Hay 2008). Infants who grow poorly have an extended hospital stay, are more prone to infection and are more likely to be re-admitted. However, the most serious long term problem is poor cognitive function. Ehrenkranz and colleagues (2006) studied preterm infants born 501-1000 grams

and separated weight gain into quartiles. When compared with infants in the quartile with the greatest rate of weight gain (21 g/kg/day), infants with the slowest weight gain (12 g/kg/day) were more likely to have neurological impairment at 18-22 months corrected age. More first quartile infants had Mental and Psychomotor Development Index scores <70 and were more likely to suffer from Cerebral Palsy. Similar outcomes are reported by Latal-Hajnal and colleagues (2003). Two hundred and nineteen (219) Very Low Birth Weight infants (birthweight <1250g) were assessed for growth and neurodevelopment at two years of age. Infants born appropriately grown for their gestational age who experienced growth failure and were <10th centile at two years of age showed the lowest Mental and Psychomotor Developmental Index scores. The second worst performing group were infants who were born in and remained in <10th centile at two years of age. Our data also supports these indings. Belfort and colleagues (2011) examined growth outcomes in conjunction with neurological function and showed that growth in the period before term, i.e. the in-hospital period, was most influential on mental development scores at 18 months corrected age. Greater weight gain (2.4 MDI points per z-score, 95% CI 0.8, 3.9, p=0.003; 2.7 PDI points, 95% CI 1.2, 4.2, p=0.0004), BMI gain (1.7 MDI points, 95% CI 0.4, 3.1, p=0.01; 2.5 PDI points,95% CI 1.2,3.9, p=0.0003) and head growth (1.4 MDI points, 95% CI -0.0,2.8, p=0.05; 2.5 PDI points, 95% C1.2,3.9, p=0.0003) were associated with higher scores. Growth from term to four months corrected age was associated with some improved Psychomotor but not Mental Development Index scores. There was no association between Mental Development Index scores and growth in the 4-12 months corrected age period.

The findings of these studies highlight the importance of growth during the in-hospital period. Quality nutrition in this critical window is vital to good neurodevelopmental outcomes. The consequences of poor neonatal growth are lifelong and every effort must be taken to ensure growth does not falter in this critical early period.

Protein is vital to growth

Growth of lean body mass (muscle, bone, brain and organ mass) is completely dependent on protein. Such structures are built on a protein matrix (Hay 2009). Fat can only make fat cells bigger, it cannot make new cells.

The fetus receives protein in the simple form of amino acids which are actively transported across the placenta at a greater rate than required for growth (Thureen and Heird 2005). Studies in fetal sheep suggest that the protein synthetic rate is two to fourfold greater than the fractional rate of growth (Meier, Peterson et al. 1981). The excess is oxidised and used as an energy source (Thureen and Heird 2005).

How much protein is necessary to allow adequate growth?

The current recommendations for protein intakes vary with gestation. The most recent guidelines were developed by the European Society of Paediatric Gastroenterology, Hepatology and Nutrition Committee on Nutrition (Agostoni, Buonocore et al. 2010). For infants less than 1000g a protein intake of 4-4.5g/kg/day at 3.6-4.1g/100kcal is recommended. For infants weighing1000-1800g, 3.5-4g/kg/day of protein at 3.2-3.6g/100kcal is recommended.

Most studies of protein content of enteral feeds have been conducted using formula. The most recent Cochrane review (Premji, Fenton et al. 2006) suggests that protein levels of formula should not exceed 4g/kg/day and remain equal to or higher than 3g/kg/day, unless it forms part of an experimental study. A study not included in the Cochrane Review found

a high protein preterm formula (average protein intake 4.6 g/kg/day) was well tolerated by infants, who did not experience any episodes of uremia or metabolic acidosis. Additionally, infants on the high protein formula, 3.6g/100 kcal, showed increased weight gain (Cooke, Embleton et al. 2006).

Breast milk is best for the preterm infant if it is fortified

The evidence for human milk being the optimal diet for term born infants is substantial (Kramer, Chalmers et al. 2001; Callen and Pinelli 2005; Tsang, Uauy et al. 2005). Breast milk for preterm infants is associated with better host defences and neurodevelopmental outcomes (Tsang, Uauy et al. 2005). Infants are able to digest and absorb nutrients more efficiently and show better gastrointestinal function (Tsang, Uauy et al. 2005) when compared to formula. Preterm infants are more difficult to feed than term infants due to gastrointestinal, suck-swallow reflex and metabolic immaturity, though they can receive adequate nutrition from breast milk if appropriate adjustments are made.

As with term breast milk, preterm breast milk shows a decline in protein concentration over time (Tsang, Uauy et al. 2005). While the protein content in the initial days of preterm breast milk may be adequate to meet the needs of the infant, as the protein content decreases the infant will experience nutritional deficit

In order to meet the needs of the preterm infant while using the known advantages of breast milk, fortifier is added to provide additional protein, energy and minerals necessary for growth. Fortifying human milk is associated with better rates of weight gain and protein accretion and the process is practiced in neonatal nurseries world-wide (Kuschel and Harding 2004). The current Cochrane review (Kuschel and Harding 2004) suggests there is no reason to further compare fortified human breast milk to non-fortified human breast milk.

A leader in the field, William Hay proposes increasing the protein concentration of human milk fortifier based on evidence provided by the Arslanoglu study (Arslanoglu, Moro et al. 2006). Arslanoglu and colleagues used an adjustable breast milk fortification feeding scheme guided by periodic determinations of blood urea nitrogen (BUN). At the highest level of fortification, protein intake averaged 3.4g/kg/day at 2.3g/100ml. Infants gained more weight per day and showed greater head circumference at discharge. All BUN levels remained in the normal range. Furthermore, they tested the hypothesis that the protein content of preterm breast milk is known to be higher than that of term breast milk but is often overestimated (Arslanoglu, Moro et al. 2009). This has grave consequences for the preterm infant who even with fortification is receiving far less protein than estimated. Assuming the protein content of breast milk given to infants to be 1.5g/100ml, they calculated the protein intake based on real feed data. When compared to the actual protein intake they found significant differences for all three weeks of the study. This data has alarming implications, suggesting that preterm infants are not receiving the protein intake that is being prescribed. If the amount of fortifier the infant receives is based on a false positive level this is a systemic problem in preterm infant nutrition. Indeed we recently found the protein content of preterm breast milk to be 1.27g/100mL (range 0.59-2.92g/100ml). There is a wide range not just between women but within each woman. Therefore it is important to fortify to the lowest level of protein content to ensure all infants are receiving adequate protein as suggested by Hay (2009). While clinical trials are required to test for toxicity levels, he stresses that without adequate protein ‘infants have no chance for improved growth’.

A study conducted within this research group by Jacqueline Miller PhD hypothesised that increasing the protein content of human milk fortifier would result in increased length for infants born <31 weeks gestation. In a blinded randomised controlled study infants were

either fed human milk fortifier (HMF) containing 1.4 g/100 ml or a standard human milk fortifier containing 1 g/100 ml.

Increasing the protein content in human milk fortifier improved length gain, the primary outcome of the study, but the difference in length gain between higher protein and control was of borderline significance (0.06cm/wk, 95% CI -0.01-0.12; p=0.08). Importantly, significantly fewer infants were classified SGA for length at discharge in the higher protein group. At birth 7 (14%) infants in the standard group and 7 (16%) infants in the higher protein group were SGA for length. At discharge 31 (63%) infants in the standard group and 21 (49%) infants in the higher protein group were SGA for length. While there was no difference at birth between the groups, significantly less infants were classified SGA at discharge in the higher protein group (p=0.04). Weight at discharge was also increased; however the rate of weight did not differ between the groups.

These are promising data, suggesting that the higher protein content encourages improved growth. To our knowledge this is the highest protein concentration of HMF for preterm infants, however we speculate the protein content was still insufficient for the growth needs of preterm infants.

It was important to monitor the safety of the dose and any adverse outcomes. Blood urea levels were higher in the high protein group as expected. Of the 43 infants in the high protein group seven developed transient uraemia (urea >8 mmol/l) and all resolved spontaneously without intervention. Conversely 15 infants (11 in the standard group) recorded low urea concentrations suggesting possible protein malnutrition.

Encouraging direct breast feeds in important

As infants mature more feeds are taken directly from the breast. This is encouraged to support the transition from tube to breast feeds with the goal of fully feeding at the breast by discharge home. Over the length of hospital stay, direct breast feeds make up an increasing proportion of infants' intakes. For example in our recent review of nutritional practices in 138 infants born <33 weeks gestation, by 7 days of age, four infants (3%) were having one feed at the breast; by 3 weeks of age, 41 (30%) were feeding directly from the breast, with a median of two feeds per day (range 1–3) (Collins et al 2008). Given that preterm infants typically have 8 feeds per day then up to 25% of their intake is not fortified. This is a contributing factor to the poorer growth and neurodevelopment outcomes of preterm infants who are breast fed compared with infants who are formula fed (Lucas, Morley et al. 1989).

There are anecdotal reports of clinicians adopting varied practices to address the lack of fortification of direct breast feeds including advising women to express, fortify feeds then feed the breast milk by bottle, or to alternate breast feeds with formula feeds. Neither of these approaches promotes the act of breast feeding. In this study we will use a novel approach to fortifying feeds by administering a low volume fortifier solution as an 'entrée' immediately prior to a scheduled breast milk feed.

Summarising the current literature on preterm infant nutrition exposes a sizable gap in the knowledge base. There is an urgent need for in-hospital based randomised controlled trials with repeated growth measurements. Good quality growth in this time is necessary for metabolic and neurological health in adult life. Breast milk is optimal for infants but it must be correctly fortified to provide the nutrients the preterm infant requires. Direct breast feeds should be encouraged despite the infant not receiving fortified breast milk for that

feed. We propose a small volume, high protein human milk fortifier 'entree' which will precede the infant's normal feed. This approach allows the researchers to accurately calculate the amount of protein the baby is receiving and ensures that when the infant moves to direct breast feeds, adequate nutrition is maintained.

5. **Preliminary Study** (if any)

Nil

6. **Participants**

60 infants born <33completed weeks gestation whose mothers plan to breastfeed will be enrolled from the Women's and Children's Hospital (WCH) Neonatal Intensive Care Unit (NICU). Infants will be randomised to one of two groups: high protein fortifier (n=30) or standard fortifier (n=30).

7. **Selection Exclusion Criteria (specific).** Also withdrawal criteria, if applicable.

Inclusion criteria. Infants 28 to <33completed weeks gestation whose mothers intend to provide breast milk and have the written informed consent of their parents/guardians.

Exclusion criteria. Infants with major congenital or chromosomal abnormalities known to affect growth or where protein therapy is contraindicated e.g. major heart defects, cystic fibrosis, phenylketonuria, disorders of the urea cycle. Infants likely to transfer to remote locations where weekly blood tests are unable to be performed.

Withdrawal – Infants can be withdrawn at any time from the study at the parents' request. Similarly, the infant can be withdrawn from the study at the discretion of the attending physician if there is concern with the health of the infant whether due to the intervention or unrelated

8. **Plan and Study Design** (including methodology, statistical analysis)

8.1 **Study design.** This is a randomised controlled trial of two parallel treatment groups to be conducted in the Neonatal Intensive and Special Care Units of the Women's and Children's Hospital. Participants, care providers, outcome assessors and data analysts will be blinded to randomisation group.
Infants will be randomly assigned to one of 2 treatment groups:

- Group 1: Infants will be fed a high protein human milk fortifier containing 1.8 g protein/100 ml of expressed breast milk.
- Group 2: Infants will be fed a standard protein human milk fortifier containing 1.0 g protein/100 ml of expressed breast milk (current clinical practice).

Infants will receive treatment from enrolment until hospital discharge.

Randomisation - sequence generation. A computer generated randomisation schedule using variable block design will be generated by statisticians independent of study conduct. Stratification will occur for sex and gestational age <29 weeks and 30to 32 weeks.

Allocation concealment and implementation of randomisation. The parent/s of eligible infants will be approached to enter the trial by the study neonatologists (CIC or CID) or nominee; follow-up for consent will be by the study research nurse. This will occur when the infant is nearing readiness to commence feeds. Upon consent, infants will be randomised to one of four colour groups and assigned a unique study ID. Each dietary

treatment (high protein group, standard protein group) will be split into two coded groups (hence a total of four coded groups). This is to reduce the risk of un-blinding.

The primary outcome is weight gain (g/day) and secondary outcomes include length and head circumference gain, SGA for weight, length and head circumference; weight, length and head circumference at discharge or expected date of delivery (whichever occurs first) and body composition.

8.2 **Dietary intervention.** The study fortifiers have been designed to be virtually identical in composition with the exception of their protein concentration. As energy concentration is known to influence growth, we have endeavoured to ensure that the fortifiers are isocaloric with a similar nutrient profile. The study fortifiers are based on a currently available commercial human milk fortifier (FM-85, Nestle). By adding either a protein powder (Protifar, Nutricia) or a carbohydrate powder (Polyjoule, Nutricia) we have produced fortifiers that differ only in their protein and carbohydrate content. Both Protifar and Polyjoule are nutrition support products commonly used in paediatrics. Glucose polymers such as Polyjoule are already an ingredient in commercial HMF. Neonatologists and dietitians currently supplement preterm infants' feeds with Protifar or Polyjoule on an 'as needs' basis.

High protein fortifier group: Infants in this group will receive a human milk fortifier (FM-85, Nestle) that is fortified with Protifar (Nutricia) to provide 1.8 g protein and 18 kcal/100 ml of milk (25kcal/30ml) of breast milk when mixed.

Standard fortifier group: Infants in this group will have a human milk fortifier (FM-85)that is equivalent to standard care and will provide 1 gram protein. This will be made isocaloric with the addition of a glucose polymer (Polyjoule, Nutricia) to provide 18 kcal/100 ml of milk (25kcal/30ml) of breast milk when mixed.

The study products will be supplied in 400 gram cans with a 12 month shelf life. Once opened tins will be discarded after one month. The cans will be named 'human milk fortifier' and will be differentiated by colour-coded labels.

8.3 **Administration of fortifier:** The study product, either Protifar or PolyJoule, will be added to HMF and mixed with sterile water (4.9grams total powder to 6mLs water which displaces to a total of 8mls) by Food Services personnel trained in the safe handling of food preparation and precise weighing and measuring techniques. Sufficient fortifier solution for a 24 hour period will be prepared, decanted into syringes with the correct amount needed for each feed, labelled and stored in the refrigerator. Any unused syringes will be discarded after 24 hours. The amount required per feed will be ordered on the neonatal drug chart as follows:

Date	Medication Trial human milk fortifier **Do not administer if formula >50% of feed**	
Calculations	Weight, kg	
	mls/kg/d	
	mls/day	
	mls fortifier/day *(0.08 x mls/day)*	
	number feeds/day	
	mls/feed *(mls fortifier/number feeds)*	
Route **ORAL**	Dose _____ mls	Frequency 3 hourly with feeds
Prescriber Signature	Print Name	

The fortifier solution will be given immediately preceding all scheduled enteral feeds (ie tube, breast or bottle) via the feeding tube. If on continuous feeds the fortifier solution will be administered as a bolus two hourly. If the infant is to receive a feed that contains >50% formula, no fortifier solution will be given. If the infant no longer requires a feeding tube then the fortifier will be gently and slowly syringed into the side of the mouth.

Expressed breast milk will not be fortified. The fortifier is only given as a solution immediately preceding feeds.

If the infant's feeds are withdrawn for any reason, the fortifier will also be ceased.

Duration of dietary intervention. The entree feedings will begin when the infant is consuming an enteral intake of ~80ml/kg/day and continue to discharge. After commencement of fortifier, enteral intake will be increased, as tolerated, until 170 to 180 mls/kg/d as per current clinical practice.

Compliance. A research nurse will monitor medication charts daily to confirm study fortifier has been given. The dose calculation will be checked twice a week against the current weight of the infant and volume of feeds and the order will be adjusted as needed.

Education of attending staff. In-service education will be held for nurses and medical staff, including the prescribing and administration of the study emulsion.

Clinical management of participants. The clinical management of participants will be under the direction of the attending neonatologist. The data obtained from the study will be available to the attending neonatologist.

Blood urea nitrogen (BUN) and metabolic acidosis. After discussion with neonatologists and for the purposes of this study population uraemia is defined as BUN levels above 8.0 mmol/L.

Infants on full enteral feeds who develop a BUN >8.0 mmol/L and/or a metabolic acidosis (base excess <-6) will have the BUN and blood gas analysis repeated within 48 hours and attending clinician notified.

If BUN remains elevated and
1) is associated with acidosis then the study fortifier will be discontinued and infant commenced on S26-SMA fortifier (1g protein/100 ml) for 48 hours and managed as clinically indicated. If acidosis is corrected, the infant may continue in the study and blood tests will monitored twice weekly for one week.

2) is not associated with other abnormal blood tests the infant may remain in the study with BUN and chemistries monitored twice weekly until returns to normal.

Infants who have poor weight gain (defined as <15 g/kg/day over the preceding 7 day period) associated with a BUN <2 mmol/L once full enteral feeds have been reached will be assessed by the attending neonatologist. Feeds will be increased to 170 to 180 mls/kg/day. If weight gain does not improve then additional protein supplements (Protifar) may be added at the discretion of the attending neonatologist. Once a weight gain of 15g/kg/day is achieved and BUN levels ≥2 mmol/L additional protein will be ceased.

8.4 **OUTCOME ASSESSMENTS**

Primary outcome

The primary outcome of the study is weight gain (g/d) from commencement of study fortifier until discharge home or Estimated Delivery Date (EDD) whichever occurs first.

Infants will be weighed daily by clinical staff while in NICU and twice weekly while in SCBU (excluding days on which research nurses weigh infant). Weight will be taken by research personnel at trial entry and then weekly while in NICU and SCBU. Measurements by research nurses will be double checked to ensure an accuracy of ± 5 grams, if >5grams a third measurement will be taken. The scales are calibrated annually using standard weights.

Secondary outcomes

Duplicate measures of recumbent length will be determined in the supine position to the nearest 0.1 cm using a recumbent length board. A third measurement will be taken if there is a discrepancy of more than 5 mm.

Head circumference will be measured in duplicate around the largest occipito-frontal circumference using a non-stretching tape. A third measurement will be taken if there is a discrepancy of more than 5 mm. Length and head circumference will be taken weekly.

SGA: Infants measuring <10^{th} percentile at time of discharge/EDD for weight, length or head circumference will be designated SGA.

Body composition: Lean body mass will be obtained by Bioelectrical Impedance.

Feeding tolerance: The number of days on which one or more feeds have been stopped and the number of days taken to reach full enteral feeds (enteral intake ≥ 150 mls/kg/day) will be recorded.

Laboratory analyses: Protein safety and use will be assessed by weekly blood (0.05 ml) and urine (1 ml) while in NICU and fortnightly in SCBU – these will be coordinated with routine tests to measure:

- blood urea nitrogen
- serum albumin
- plasma creatinine
- total protein
- blood ph, bicarbonate and base excess
- whole blood amino acids
- urinary amino acids

Analysis of expressed breast milk: A weekly sample (8 mL of pooled 24 hour milk) will be collected and frozen for later analysis of the protein content of the milk. Analysis will be done using an infra-red analyser. The protein content of human milk varies both between individuals and within the same individual over time. Mothers of preterm infants have a higher level of protein in their milk, declining over time. In the clinical setting, milk is generally fed sequentially according to date expressed except when fresh milk is available, where it is preferentially used. This may mean that surplus milk from earlier in the postnatal period which has higher protein content may be defrosted and fed at a later date. This analysis will enable us to more accurately determine the total protein intake for each week.

Other clinical outcomes: Apgar scores, grade of intra-ventricular haemorrhage (IVH), confirmed sepsis, confirmed necrotising enterocolitis, grade of retinopathy of prematurity, days of corticosteroid use, days of oxygen therapy, oxygen at 36 weeks post menstrual age.

8.5 **Study conduct**

At enrolment. Informed consent**;** record demographic and baseline characteristics - infant sex, birth weight, gestational age, date of birth, multiplicity, age at recruitment into study, anthropometric measurements at recruitment, age and parity of mother; randomisation to study group; prescribe study on drug chart (CIC McPhee or CID Stark or nominee); alert Food Room personnel to new enrolment.

Daily. Weight by clinical staff as per standard care (when in NICU, twice weekly in SCBU)
Feed tolerance observations by research personnel.

Weekly. Weight, length and head circumference measurement by research personnel; lean mass measurement using bioelectrical impedance spectroscopy; 500 μL blood sample via heel prick; 1 ml urine sample; 8 ml Breast milk sample.

8.6 **Statistical considerations**

Sample size. We aim to include 30 infants per group giving a total sample size of 60 infants.
With a sample size of 30 per group it will be possible to detect a difference of 3.31 grams per day between the high protein and standard protein groups with 80% power and P=0.05.

Data analyses. Data will be analysed using a repeated measures model in which clustering due to repeated measures and multiple births will be accounted for. Analyses will be performed on data from all babies randomised on an intention to treat basis and probability <0.05 will be considered significant.

A per protocol analysis will be undertaken and will exclude data from infants where breast milk made up ≤70% of total enteral intake over study period.

Study management. The study steering committee will consist of all Chief Investigators, chaired by CIB, and will meet fortnightly to monitor study progress.

Time line. We aim to recruit two infants per week, achieving sample size in 30 weeks. Recruiting is anticipated to commence in December 2011, the last infant will complete the study therefore in October 2012.

8.7 Study approval and conduct

Regulatory Approval. As human milk fortifier, Protifar and PolyJoule are not considered therapeutic goods by the TGA they have informed us that there is no need to notify them by the CTN scheme. However if the committee deems it necessary we will summit a CTN to the TGA. Please see the attached PDF for email correspondence from TGA senior pharmacist Josephine Dufty.

Ethical Considerations. This study will be carried out in accordance with the Australian National Statement on Ethical Conduct in Research Involving Humans. All data collected will be treated with confidence and parents/guardians will be free to withdraw their infants from the study at any time, without explanation and without prejudice to their future care.

Written Informed Consent. All parents/guardians will have the study explained by a Chief Investigator or nominee. They will receive a full explanation, in lay terms, of the aims of the study as well as the discomfort, risks and benefits of participation. The study is for research purposes and any therapeutic benefit to the individual is unknown. The parents' right to withdraw from the study at any time without prejudice will be confirmed. The parent/guardian will be required to provide written informed consent and will be given a copy of the signed Consent Form.

9. Therapeutic Substances

Dose, route of administration, formulation. The human milk fortifier to be used in this trial are based on a currently available commercial fortifier (FM85, Nestle, Switzerland, nutritional composition, Appendix A) to which is added a protein powder (Protifar, Nutricia, nutritional composition Appendix B) or a glucose polymer (PolyJoule, Nutricia, nutritional composition Appendix C) to attain the desired protein and energy levels. The two fortifiers will be isocaloric and have similar micronutrient content. Composition is as follows:

Group 1. High Protein, Standard Energy

Per 100 ml breast milk

	HMF	**Protifar**	**PolyJoule**	**Total**
Amount g	4	0.9	0	4.9
Protein	1	0.8	0	1.8
kcal	**14.6**	**3.42**	**0**	**18**
Kcal/30ml breast milk	**4.4**	**1**	**0**	**5.4**

Group 2 Standard protein, Standard energy

Per 100 ml breast milk

	HMF	**Protifar**	**PolyJoule**	**Total**
Amount g	4	0	0.9	4.9
Protein	1	0	0	1.0
kcal	**14.6**	**0**	**3.42**	**18**
Kcal/30ml breast milk	**4.4**	**0**	**1**	**5.4**

Monitoring – therapeutic response. The expected therapeutic response to the increased protein study fortifier is an increase in growth. This is being monitored by measuring weight gain, length and head circumference.

Monitoring - potential adverse effects, reporting of adverse effect. The protein level in the high protein group is within the recommended range for preterm infants when calculated according to lower protein content breast milk. The level of protein to be administered is similar to levels used in a recent clinical trial which reported no adverse effects from the high protein group (Cooke, Embleton et al. 2006). Nevertheless monitoring of the infants response to the increased protein load is essential.

Adverse Events: Records of adverse events will be collected and include any untoward medical experience whether or not it is considered related to the study. These include:

- Incidence of Necrotising Enterocolitis (NEC)
- Sepsis
- Surgery
- Death

Adverse events will be reported to DTC, REC and TGA within the expected REC timeframe as they occur and also as an annual report.

10. Safety & Ecological Considerations

Not applicable

11. **Ethical Considerations.** This study will be carried out in accordance with the Principles of International Conference on Harmonisation (ICH) Good Clinical Practice (GCP) (as adopted in Australia) which builds upon the ethical codes contained in the current version of the Declaration of Helsinki and the Australian National Statement on Ethical Conduct in Research Involving Humans.
All data collected will be treated with confidence and parents/guardians will be free to withdraw their infants from the study at any time, without explanation and without prejudice to their future care.

12. **A Specific Consent Form and Information Sheet should be included**.

Notes:

- *For participants who are under 18, parental/guardian consent must be obtained. If parents/guardians have consented to their child's participation, then consent should also be obtained from those children/young persons deemed sufficiently mature.*

- *Research involving children or people with a mental illness or intellectual disability which would preclude consent, should only be conducted when it is in their best interests.*

- *Parents must be aware that can withdraw their child from the study at any stage and that this will not affect the medical care or any other aspects of their child's relationship with the relevant healthcare services.*

13. **Confidentiality and Data Security**. A description of the information to be collected, the sources used, and the safeguards to preserve the confidentiality of the

data should be listed. This should include the means of data storage (e.g. computer files).

The participants will be assured that confidentiality and anonymity will be strictly respected and maintained, and that they will not be identified in the study. Data entry will be de-identified and kept in a password protected area. Hard copy data will be kept in a locked office within the Child Nutrition Research Centre. On final completion of the study and after publication of results data will be stored in a secure off-site location for 30 years according to the Department of Human Services 'Records Disposal Schedule for South Australian Public Hospital' (NO.2000/0012).

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Nutritional Composition of FM-85 (Nestle)

		Per 1 gram
Energy	kcal	3.5
Fat	g	0.004
Protein	g	0.2
Carbohydrate	g	0.7
Sodium	mg	5.2
Potassium	mg	13
Chloride	mg	4.6
Calcium	mg	15
Phosphorus	mg	9
Magnesium	mg	0.8
Manganese	µg	1.3
Selenium	µg	0.5
Iron	mg	0.3
Iodine	µg	2.6
Copper	mg	0.01
Zinc	mg	0.2
Vitamin A	µg	71
Vitamin D	µg	0.8
Vitamin E	mg	0.8
Vitamin K	mg	1.6
Vitamin C	mg	3.5
Vitamin B1	mg	0.03
Vitamin B2	mg	0.04
Niacin	mg	0.3
Vitamin B6	mg	0.03
Folic Acid	µg	8.0
Pantothenic acid	mg	0.1
Vitamin B12	µg	0.02
Biotin	µg	0.7
Choline	mg	1.7
Inositol	mg	0.8
Taurine	mg	0.4
Carnitine	mg	0.7

Nutritional Composition of Protifar (Nutricia)

		per 100g
Energy	kcal	380
Protein Equivalent	g	89.0 (93%E)
Casein	g	71.2
Whey Protein	g	17.8
Nitrogen	g	13.9
NPC:N ratio		
Carbohydrate	g	<1.5 (2%E)
as Lactose	g	<1.5
Sucrose	g	
Fat	g	<2.0 (5%E)
Saturates	g	1.5
Monounsaturates	g	0.5
Polyunsaturates	g	0.1
Fibre	g	0
Water	g	<5
Sodium	mg	100
Potassium	mg	120
Calcium	mg	1350
Phosphorus	mg	700
Magnesium	mg	<20
Chloride	mg	100

Nutritional Composition of PolyJoule (Nutricia)

		per 100g
Energy	kcal	384
	kJ	1630
Protein Equivalent	g	–
Carbohydrate	g	95 (100%E)
as Lactose	g	0
as Sucrose	g	0
Fat	g	0
Fibre	g	0
Water	g	<5
Sodium	mg	<5

Appendix B - Participant screening log

POPPET study weekly screening and tracking log

Week beginning:			**Screening criteria -must be yes**					**Approach/consent**			**Comments**
Name		**UR Number**	Inclusive of 32.6 and 28.0 weeks gestation	Date of standard fortification beginning	Less than 4 consecutive days of commencing **fortfied** feeds	Free of major congenital/ chromosomal abnormality	Unlikely to be transferred to rural/remote location	Eligible (Y/N)	Approached (Y/N) / Dat	Consent (Y/N)	**Include:** **Reason not approached** (enter reason) **Reason for non consent** (enter one or more of following numbers): 1. Partner or other family member does not want to participate 2. Does not want to take part in any research 3. Does not want to be randomised 4. Other (please state) **Reason if consented but not randomised** (enter reason) **Any other comments**
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											

Appendix C - Parent information sheet

Government of South Australia

Children, Youth and Women's Health Service

Protein intake and growth in premature babies

Scientific title: *Does increasing the protein content of human milk fortifier improve growth in preterm infants <33 weeks gestation? A randomised controlled trial.*

You are invited to take part in a study to help determine whether increasing the amount of protein that is added to breast milk feeds improves the growth of premature babies. A new approach to giving the protein will be used, allowing all feeds to be fortified. This study is being undertaken by Ms Jessica Reid as part of a PhD at The University of Adelaide and is supervised by Dr Carmel Collins, Dr Andy McPhee and Prof Maria Makrides.

Protein and expressed breast milk

Protein is vital to growth at all stages of life but it is especially important to premature babies. Breast milk is best for all babies however for premature babies, breast milk needs protein added to it (in the form of a 'human milk fortifier') for the baby to grow well. This is standard practice at WCH.

We recently found that giving a little more protein to premature babies marginally improved their growth and was safe. It is hoped that this study will determine the level of protein in human milk fortifier to achieve best growth.

What do we want to find out with this study?

We want to see if giving premature babies more protein will improve growth.

New approach to giving human milk fortifier

As premature babies grow and mature they progress from having feeds given by a tube to breast or bottle feeds. With current practice when feeding directly at the breast the milk is not fortified. In this study the fortifier will be given by the tube immediately before a feed so that all breast milk feeds whether given by tube, breast or bottle are fortified.

What does the study involve?

Your baby will be randomly assigned (like tossing a coin) to one of two groups. One group will receive a human milk fortifier containing a standard level of protein and the other group a human milk fortifier with a higher level of protein. In both groups the fortifier will be given immediately before a feed through the feeding tube.

Neither you nor the research or clinical team will be able to choose which group you are in or know which fortifier your baby will be given. Your baby will continue to receive the study fortifier until discharged home. Should your baby need formula, s/he will receive the standard preterm formula used in the neonatal unit regardless of which group they are in. Your baby will receive the fortifier solution

before every feed of breast milk (tube, breast or bottle). If more than half of a feed is formula the study solution will not be given.

The levels of breast milk fortifier protein in the study are similar to the range seen in many infant formulas and are unlikely to put your baby at risk. Nevertheless we will take small blood and urine samples to monitor how proteins are metabolised (used) by your baby. Where possible we will use information from the routine blood tests your baby has as part of the daily care. We may need to take an additional blood test once a week.

What will happen during the study?

1. Your baby will be weighed daily in NICU and twice a week in SCBU

2. At enrolment and at weekly intervals until your baby leaves the hospital, his/her weight, length and head circumference will be measured. These will be done again on the day of discharge from hospital.

3. Every week and near discharge, body composition will be measured to get an estimate of the proportion of lean and fat mass. Two small leads are placed on the hand and two on the foot and it takes ten seconds to get the readings. There is no discomfort with this procedure and it can be done while your baby is sleeping.

4. Feeding and health information will be taken from your baby's medical records.

5. A weekly blood test (0.5 ml) will be taken by heel prick to measure protein in your baby's blood. These will coincide with routine blood tests whenever possible. They will be taken by experienced personnel.

6. A weekly urine test will be collected to measure amino acids (proteins) in the urine. Only a small spot of urine is needed and this will be collected on cotton wool inside the baby's nappy. This will not cause any discomfort.

7. A small sample of expressed breast milk (8 ml) will be taken once a week to measure the protein content of the milk. This will only be taken if surplus to your baby's needs.

The study nurse will also review your baby's medical records to document details regarding the birth and feeding progress. The study nurse may also need to review your medical records for additional information about your baby's birth.

Risks and benefits of the study

There may be no benefits to your baby. However, if your baby is randomised to the high protein group, they may have better growth. There are no known risks of increasing the protein to the levels that we are using in this trial. However, the weekly tests will monitor the protein in your baby's blood and urine. If your baby appears to be unwell for any reason standard hospital protocols will be enforced. If the baby's doctor thinks the baby is unwell because of the study your baby will go to normal feeding. Blood tests will cause some temporary pain and may cause a short term bruise. This can be minimised in neonates by giving them a small amount of sucrose in their mouth as is standard nursery practice.

Your rights

Your participation in this study is voluntary. If you do not wish to take part in the study your baby will receive standard clinical care. You are free to withdraw from the study at any time without explanation of why you have chosen to do so and without prejudice to you and your baby.

All information gathered will be treated with confidence except in the case of a legal requirement to pass on personal information to authorised third parties. This

requirement is standard and applies to information collected both in research and non-research situations. Such requests to access information are rare; however we have obligation to inform you of this possibility. No information that could identify you or your baby will be released to any person not associated directly with the study. The results of this trial may eventually be published in medical journals and presented at professional meetings, but you or your baby will not be identified in any way.

Any questions?

This study has been reviewed by the Women's and Children's Health Network Research Ethics Committee. Should you wish to discuss the study with someone not directly involved, in particular in relation to matters concerning policies, or your rights as a participant, or should you wish to make a confidential complaint, you may contact the executive secretaries of the committee, Ms Brenda Penny, WCH, 8161 6521. If you wish to discuss with someone directly involved in the study please contact:

Jessica Reid 8161 6848
Dr Andrew McPhee 8161 7631.
Prof Maria Makrides 8161 7443
Dr Carmel Collins 8204 5755
Dr Michael Stark 8161 763

Appendix D - Consent form

WOMEN'S AND CHILDREN'S HEALTH NETWORK HUMAN RESEARCH ETHICS COMMITTEE

CONSENT FORM

LAY TITLE
Protein intake and growth in premature babies

SCIENTIFIC TITLE

Does increasing the protein content of human milk fortifier improve growth in preterm infants <33 weeks gestation? A randomised controlled trial.

I _____

hereby consent to my child's involvement in the research project entitled:

"Does increasing the protein content of human milk fortifier improve growth in preterm infants <33 weeks gestation? A randomised controlled trial."

1. The nature and purpose of the research project described on the attached Information Sheet has been explained to me. I understand it and agree to my child taking part.

2. I understand that my child may not directly benefit by taking part in this study.

3. I acknowledge that the possible risks and/or side effects, discomforts and inconveniences, as outlined in the Information Sheet, have been explained to me.

4. I understand that I can withdraw my child from the study at any stage and that this will not affect medical care or any other aspects of my child's relationship with this healthcare service.

5. I understand that there will be no payment to my child for taking part in this study.

6. I have had the opportunity to discuss taking part in this research project with a family member or friend, and/or have had the opportunity to have a family member or friend present whilst the research project was being explained by the researcher.

7. I am aware that I should retain a copy of the Consent Form, when completed, and the Information Sheet.

8. I consent to my baby having the following procedures:
   - Regular growth measures while my baby is in hospital.
   - A weekly blood test (which will be coincided with routine blood tests where possible) until discharge or estimated delivery date.
   - Weekly urine sample.
   - Weekly body composition measurements.

9. I consent to providing a small sample of breast milk (8 mls) once a week if possible for analysis of the protein and energy content

10. I understand that I am free to stop donating breast milk and my infant's blood samples at any stage, without giving any reason, and that my action of donating/not donating a sample will not affect (i) my prospects in any position; or (ii) any other conceivable situation.

11. I understand that study personnel will review my baby's medical records at the Women's and Children's Hospital and any other hospital my baby may be transferred to and from, for the duration of the study.

12. I understand that study personnel may review my medical records.

13. I am aware that I may be contacted in the future about related research studies.

13. I understand that my (my child's) information will be kept confidential as explained in the information sheet except where there is a requirement by law for it to be divulged.

Signed: .....

Relationship to patient: .....

Full name of patient: .....

Dated:.....

I certify that I have explained the study to the parent and consider that she/he understands what is involved.

Signed: ..... Title: .....

Dated: .....

Appendix E - Nutritional content of study products

Nutritional Composition of FM-85 (Nestle)

		Per 1 gram
Energy	kcal	3.5
Fat	g	0.004
Protein	g	0.2
Carbohydrate	g	0.7
Sodium	mg	5.2
Potassium	mg	13
Chloride	mg	4.6
Calcium	mg	15
Phosphorus	mg	9
Magnesium	mg	0.8
Manganese	µg	1.3
Selenium	µg	0.5
Iron	mg	0.3
Iodine	µg	2.6
Copper	mg	0.01
Zinc	mg	0.2
Vitamin A	µg	71
Vitamin D	µg	0.8
Vitamin E	mg	0.8
Vitamin K	mg	1.6
Vitamin C	mg	3.5
Vitamin B1	mg	0.03
Vitamin B2	mg	0.04
Niacin	mg	0.3
Vitamin B6	mg	0.03
Folic Acid	µg	8.0
Pantothenic acid	mg	0.1
Vitamin B12	µg	0.02
Biotin	µg	0.7
Choline	mg	1.7
Inositol	mg	0.8
Taurine	mg	0.4
Carnitine	mg	0.7

Nutritional Composition of Protifar (Nutricia)

		per 100g
Energy	kcal	380
Protein Equivalent	g	89.0 (93%E)
Casein	g	71.2
Whey Protein	g	17.8
Nitrogen	g	13.9
NPC:N ratio		
Carbohydrate	g	<1.5 (2%E)
as Lactose	g	<1.5
Sucrose	g	
Fat	g	<2.0 (5%E)
Saturates	g	1.5
Monounsaturates	g	0.5
Polyunsaturates	g	0.1
Fibre	g	0
Water	g	<5
Sodium	mg	100
Potassium	mg	120
Calcium	mg	1350
Phosphorus	mg	700
Magnesium	mg	<20
Chloride	mg	100

Nutritional Composition of PolyJoule (Nutricia)

		per 100g
Energy	kcal	384
	kJ	1630
Protein Equivalent	g	–
Carbohydrate	g	95 (100%E)
as Lactose	g	0
as Sucrose	g	0
Fat	g	0
Fibre	g	0
Water	g	<5
Sodium	mg	<5

Appendix F - Study product preparation standard operating procedure

JOB / TASK: Poppet Study Production	DEPARTMENT: FOOD SERVICES
DATE: 29th February 2012	REVIEW DATE: February 2013

1. PRESTART CHECK

- Only trained staff can perform this task
- This is a blind study so it is important to keep product information and patient details confidential. The process is overseen by university staff and if the integrity of the study is not maintained the results of the study may be disallowed. Jess has the opportunity to demonstrate that growth and development of tiny babies can be improved by nutrition and we as a department need to support her in this.
- BABIES ON THE POPPET STUDY PROGRAM, WILL BE GIVEN HMF IN THE POPPET SYRINGES ONLY, NOT ADDED TO EBM, UNLESS SPECIFICALLY ORDERED BY THE DOCTOR OR DIETICIAN. IF WARD STAFF ORDER EBM 24 FOR A POPPET STUDY BABY, CLARIFY WITH THE WARD BEFORE YOU START PRODUCTION.
- Refer to Food Safety Plan 5.1.9 Infant Formula Preparation, for information on storage and handling OF Poppet Study product.
- Refer to the SOP Poppet Fortifier Preparation

2. OPERATION

- Four different products will be used during the study. The products will come to you in different colored unlabeled tins.
- Each tin will have a batch code and recommended use by date once opened.
- Continue our usual practice of labeling once the tin is opened
- Product will be made in bulk in the Formula Room each morning and then decanted into syringes.
- Syringes are labeled using our current label system. There is a Poppet Study page on both computers, which you will use to record production in the same way we do for the EBM.
- The Poppet Study Order sheet will give you information on what feeds are needed for the day. The order sheet will be sent to Deanna's computer week day afternoons. On Friday afternoon the lists will be sent for Sat/Sun/Mon. and be collected by FR Staff.
- Calculate how much of each product you will need to make by adding each of the required amounts for the Red babies together.

3. MAKING THE RECIPE

- EG red baby Bailey 1 5 ml, Red baby Jones requires 10 ml red baby total to make is 25 ml
- Use the SOP Poppet Fortifier Preparation Chart for quantities and mix with sterile water as per instructions

AMOUNT. REQ	MAKE UP	STERILE WATER	POPPET PROD	HMF
21-25ML	30ML	15ML	3.4G	18.8G

EG IF 25 ML ARE NEEDED FOLLOW THE 21-25 AMOUNT REQUIRED IN MILS IN THE FIRST ,

COLUMN.THIS WILL ACUTALLY MAKE 30ML OF MADE UP PRODUCT WHICH GIVES US SOME

EXTRA TO HELP WITH DECANTING AS IT IS VERY THICK

- Take care to be accurate in the weighing and decanting of product
- Product should be made using An EBM pot and stirred gently with a sterile spoon to dissolve the products, then draw up into syringes
- Prepare one feed at a time, changing gloves after each feed
- Once made the product will be decanted into 2ml or 5ml syringes, capped with a sealing cap and labeled with all relevant details.
- Product will be stored in small Décor containers labeled with the patient name and UR Number
- Continue this process for each different colored group of babies until production is complete
- Once production is complete ensure it is checked by two staff before dispatch.
- Product is then taken to the various ward fridges.
- Any old stock or empty Décor containers must be collected and returned to the kitchen for washing.
- Poppet Study product will be given as a drug by Midwives so at the end of the 24 hr period it should all have
  been used.
- If you do find significant left over product, please complete a Non- Conformance Corrective Action Report and give it to your **Team Leader who will advise Jess Reid on Ext 16848 or 0410355129, Carmel 82045755.**
- Clean and sanitize area and complete cleaning forms and save stats. when production is complete

TAKE CARE TO FOLLOW EACH STEP AND BE AWARE OF THE HAZARDS.

THIS PROVIDES A SAFE WORKING METHOD PROVIDING PROCEDURES ARE FOLLOWED.

Appendix G - Weight measurement standard operating procedure

Title: Weight Measurement	
Document ID: CNRC_004	**Version : D.V5**
Author: Carmel Collins, Jo Collins	
Effective Date:	**Review Before:**
Department/Institution Name: ***Child Nutrition Research Centre***	

Document Revision History			
Version No.	***Reason for Issue / Change***	***Date***	***QA Initial***
2	SOP format updated		CC
3	*Sub-section:* 3.4.2: Added guidelines for third measurement	16/09/2011	CC and JC
3	Minor Editing		JC and CC
4	Added: Sub section: 3.1.4: Rounding instructions Sub-section: 3.3.2: Instructions for weighing on carpet Section: 4: Training		MW
5	Amended section 3.5.2 Added 'Tared Weighing" into section 3.6 Added combined anthropometric competency checklist (appendix 1)		MW

NOTE: This document becomes an uncontrolled version once printed.

Please verify current version in electronic SOP records when reading hard copy SOP's. All Current SOP's are filed on the Shared Drive in PDF format.

1. INTRODUCTION AND PURPOSE

The objective of this SOP is to detail the procedures required when performing weight measurements on study participants involved in clinical trials within the Child Nutrition Research Centre (CNRC) or in collaboration with the CNRC.

The anthropometry procedures outlined in this SOP are based on the WHO Multi-centre Reference Growth Study and ISAK, and are a guide for measurements performed within the CNRC.

2. SCOPE/ APPLICABILITY

This SOP applies to all clinical research staff within the CNRC who are expected to perform or assist with anthropometric measurements. The anthropometry procedures outlined are a guide for measurements performed in relation to CNRC clinical trials, there may be study specific requirements for individual clinical trials which require additional procedures, please refer to your trial protocol.

3. PROCEDURE

3.1 Preparation

3.1.1 Assemble equipment required

3.1.2 Body weight needs to be measured with a direct reading electronic balance. The balances should be accurate to at least the nearest 5 grams. The accuracy of each balance needs to be checked at least two or three times yearly. Calibrated or standard weights will be available for this purpose.

3.1.3 Weight will be measured in kilograms to the nearest 10g with recumbent scales, and 100g with floor scales.

3.1.4 If scales being used weigh to more decimal points than there is room to enter in the case report form (CRF), apply numerical rounding rules to reach appropriate number of decimal points, i.e. if 5 and over then round up, if less than 5 then round down.

3.1.5 Inform the participant/parent as to what measurement is being taken.

3.1.6 Child <2 years of age should be weighed on recumbent electronic scales and weighed naked.

3.1.7 Children remove shoes and heavy outer clothing, and ideally weighed in underwear.

3.1.8 Adults remove shoes and outer clothing.

3.2 Recumbent Electronic Scales

3.2.1. Place disposable paper sheeting on the scales prior to weighing the child.

3.2.2. Children under 2 years of age should be weighed naked.

3.2.3. Zero the scale before the child is placed on the scale.

3.3 Floor scales

3.3.1. Ensure the scales are placed on a hard flat surface and the scale is zeroed before the participant stands on them.

3.3.2 If scales are being used on carpet, ensure the leg attachments are placed underneath the scales.

3.4 General principle for anthropometric measurements

3.4.1 It is ideal to complete the anthropometric profile in the order specified by each individual study, and then complete a second anthropometric profile. This may not always be possible, for e.g. with infants, where two consecutive measures are taken.

3.4.2 A third measurement is taken if the first and second measurement differ by >0.5 cm for height and circumferences and 0.1 kg for weight. (In studies involving preterm infants a third measurement is taken if the weight differs by 20 grams.)

3.5 Weight Measurement - If the child is less than 2 years old

3.5.1 Check scales have been zeroed

3.5.2 Place the infant/child on his/her back or sitting on the tray of the scale. Make sure that the infant/child is not touching anything off of the scale.

3.5.3 Wait until the child has stopped moving, then record the weight.

3.5.4 Remove the child from the scales and re-zero before performing further measurements.

3.6 Weight Measurement - In parents arms

For children unwilling to cooperate with the recumbent electronic scale, the child can be weighed in parent's arms as follows (Figure 1):

3.6.1 Ask the parent to remove shoes and any heavy clothing and stand on the zeroed electronic floor scale.

3.6.2 Make sure feet are centred in the middle of the scale.

For scales that do not tare

3.6.3 Record parent weight

3.6.4 Ask parent to step off scales, zero scales, parent and child step on scales

3.6.5 Subtract parent weight from combined parent/child to obtain child weight

3.6.6 Record weight

Tared Weighing

3.6.7 With the parent still on the scale and their weight displayed, tare the scale

3.6.8 Hand the child to the parent and ask them to remain still

3.6.9 The child's weight will appear on the display. Record the weight.

Note: If a parent is very heavy (e.g. more than 100kg) and the child's weight is relatively low (e.g. less than 2.5kg) the child's weight may not register on the scale. In such cases, have a lighter person hold the child on the scale.

Figure 1

3.7 Weight Measurement - *If the child is 2 years or older*

If the child is 2 years or older and will stand still on the electronic floor scales, weigh the child alone (Figure 2).

3.7.1 Ask the parent to help the child remove shoes and outer clothing (anything easily removable without causing discomfort; ideally weighed in underwear).

3.7.2 Ensure the scale is zeroed before the child steps on the scale.

3.7.3 Ask the child to stand in the middle of the scale, feet slightly apart and with weight evenly distributed on both feet and to remain still until the weight appears on the display.

3.7.4 The child's hands should be hanging loosely at his/her side.

3.7.5 Record the child's weight to the nearest 0.1kg.

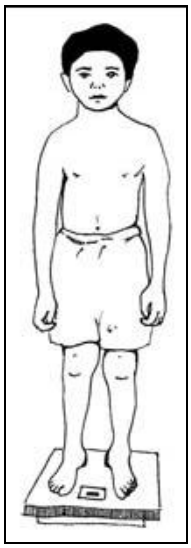

Figure 2

3.8 Care for measurement equipment

Proper care of the scales is important to ensure that measurements are as accurate as possible.

The equipment must be kept clean and stored at normal indoor temperature, protected from humidity and wetness.

3.8.1 Cleaning

3.8.1.1 Disposable paper towelling should be used to protect the scales while weighing and disposed after each use.

3.8.1.2 Scales should be wiped with a hospital grade disinfectant wipe.

3.8.1.3 Any body fluids should be cleaned immediately with a detergent and water solution.

3.8.2 Calibration

Accuracy of the scales should be checked monthly using calibrating weights and recorded on the equipment checklist.

4. TRAINING

4.1 Explain the procedure to a trained staff member.

4.2 Observe a trained staff member performing weight measurements.

4.3 Be observed performing weight measurements, as part of a complete anthropometric profile, following the method outlined in this SOP.

4.4 Trainees must be observed performing measurements on 3 participants.

4.5 The trainer must complete and sign the competency checklist (Appendix 1), which can be found at W:\CNRC\SOP's\Master SOPs\SOP Forms\Anthropometric Competency Checklist

4.6 Accuracy of the trainees measurements must also be assessed.

- **4.6.1** The trainee is to record weight measurements on the Trainee Anthropometric Training Record (Appendix 2), which can be found at W:\CNRC\SOP's\Master SOPs\SOP Forms\Trainee Anthropometric Training Record
- **4.6.2** The trainer is to repeat the measurements, recording their values on the Trainer Anthropometric Training Record (Appendix 3), which can be found at W:\CNRC\SOP's\Master SOPs\SOP Forms\Trainer Anthropometric Training Record, and intra-tester and inter-tester Technical Error of Measurement (TEM) assessed.
- **4.6.3** The trainee must achieve an intra-tester TEM of 2.0% and an inter-tester TEM of 2.5%.

4.7 Upon satisfactory completion of the competency checklist and anthropometric training record, the trainer must complete the CNRC Internal Training Record (Appendix 4), which can be found at W:\CNRC\SOP's\Master SOPs\SOP Forms\CNRC Internal Training Record.

4.8 If the trainee does not satisfactorily complete the training, the procedure must be repeated from step 4.1.

4.9 20 complete anthropometric profiles must be recorded on the Anthropometric Profiles form (Appendix 5), which can be found at W:\CNRC\SOP's\Master SOPs\SOP

Forms\Anthropometric Profiles, within the next 6 months of achievement of competency. An intra-tester TEM of 1.5% is required.

4.10 The completed and signed competency checklist, anthropometric training records, internal training record and anthropometric profiles are to be given to the Operations Manager (OM) or Document Controller (DC) for filing in the Staff Training folder.

4.11 Training is to be reviewed every 12 months.

5. GLOSSARY

Operations Manager
Manager for Operation within the CNRC, directly reports to Director of WCHRI.

Document Controller
A person responsible for the distribution and maintenance of SOP's.

6. REFERENCES

[1] WHO MGRS protocol de Onis and Food and Nutrition Bulletin, Vol 25, no 1, page 5
[2] Marfell-Jones M, Olds T, Stewart A, Carter L. *International standards for anthropometric assessment:* International Society for the Advancement of Kinanthropometry: Potchefstroom, South Africa. 2006

7. APPENDIX

Appendix 1. Anthropometrics competency checklist
Appendix 2. Trainee anthropometric training record
Appendix 3. Trainer anthropometric training record
Appendix 4. CNRC internal training record
Appendix 5. Anthropometric profiles

Appendix G cont. - Recumbent length measurement standard operating procedure

Title: Recumbent Length	
Document ID: CNRC_005	**Version : V4**
Author: Carmel Collins, Jo Collins	
Effective Date:	**Review Before:**
Department/Institution Name: ***Child Nutrition Research Centre***	

Document Revision History			
Version No.	***Reason for Issue / Change***	***Date***	***QA Initial***
1	First issue: (..\Superceded SOPs\SOP _Anthropometric Measurements_Length&Height_APPROVED_21Sep07.doc)	21/09/2007	
2	Height separated as another SOP (CNRC_SOP_015_.V3_Height Measurement). Originally was combined with Length SOP.	11/03/2009	CC
3	1. Clarification that nappies need to be removed for measurement 2. Pictures updated 3. Reference number 2 added	27/7/12	CC
4	Added section: 4. Training	31/01/2013	MW

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1. INTRODUCTION AND PURPOSE

The objective of this SOP is to detail the procedures for recumbent length measurements. These procedures should be followed for all assessments associated with CNRC clinical trials.

Standardisation in the identification of measurement sites and in measurement techniques is crucial to ensure consistent and accurate results.

2. SCOPE/ APPLICABILITY

This SOP applies to all clinical research staff within the CNRC and associated with CNRC clinical trials who are expected to perform or assist with anthropometric measurements. The anthropometry procedures outlined are a guide for measurements performed in relation to CNRC clinical trials, there may be study specific requirements for individual clinical trials which require additional procedures, please refer to your trial protocol.

Recumbent length is performed on children <2 years of age.

This procedures comply with the *World Health Organisation*[1] guidelines for recumbent length.

3. PROCEDURE

3.1 Preparation

3.1.1 Adhere to hand hygiene principles during procedure.

3.1.2 Assemble equipment required:

- Recumbent length board placed on flat surface
- Paper or electronic case report form for documenting measurements

3.1.3 Inform the parent as to what measurement is being taken and request that they prepare the infant by removing clothes.

3.1.4 Two people are required to take this measurement - explain to the parent that s/he will need to place the infant on the length board and help to hold the head in place while the measurement is taken. Child's clothes should be removed including nappy. Nappies can make it difficult to hold the infant's legs together and straighten them out fully.[2]

3.1.5 Braids or hair ornaments should also be removed if they interfere with positioning the head.

3.1.6 Cover the length board with soft disposable paper sheeting to avoid causing discomfort.

3.1.7 Ensure that the participants' privacy is maintained and that the room is a comfortable temperature.

3.1.8 Child's parent or another adult should be present

3.1.9 Two people are required to undertake a recumbent length measurement.

3.2 Length Measurement

General principle for anthropometric measurements

3.2.1 Do a complete anthropometric profile in the order specified by each individual study, and then complete a second anthropometric profile. This may not always be possible, for e.g. with infants, where two consecutive measures are taken.

3.2.2 A third measurement is taken if the first and second measurement differ by >0.5 cm.

3.2.3 Read measurement at eye level

3.2.4 Do not round up measurements as has not reached that measurement

Definition

3.2.5 Recumbent length is measured from the top of the participant's head to his or her heels while the participant is lying supine.

Method

3.2.6 Ask the parent or assistant to lay the child on their back with head against the fixed headboard, compressing the hair.

3.2.7 Quickly position the head so that an imaginary vertical line from the ear canal to the lower boarder of the eye socket is perpendicular to the board. (The child's eyes should be looking straight up). Ask the parent/assistant to move behind the headboard and hold the head in this position. (Refer figure)

3.2.8 Stand to the side of the length board to enable you to hold down the child's legs with one hand and move the foot board with the other hand.

3.2.9 Check that the child lies straight along the board and does not change position. The long axis of the child's body should be aligned with the centre line of the backboard. Shoulders and buttocks should touch the board, and the spine should not be arched. Ask the parent/assistant to inform you if the child arches the back or moves out of position. Hold down the child's legs with one hand and move the footboard with the other. Apply gentle pressure to the knees to straighten the legs as far as they can go without causing injury. *Note: it is not possible to straighten the knees of newborns to the same degree as older children. Their knees are fragile and could be injured easily, so apply minimum pressure*

3.2.10 While holding the knees, pull the footboard against the child's feet. Place gentle pressure on the child's knees to straighten the legs while positioning the footboard against the child's feet. The soles of the feet should be flat against the foot board with toes pointed directly upward. If the child bends the toes and prevents the foot board from touching the soles, scratch the soles slightly and slide the foot board quickly when the child straightens the toes. The footboard should be pressed firmly

enough to compress the soft tissues of the soles but without diminishing the vertebral column length.

3.2.11 Record the length to the nearest 0.1cm.

3.2.12 Reposition the child's trunk and check the positioning of the head and measure for a second time.

3.2.13 If the measurements differ by >0.5 cm then repeat a third measurement.

3.2.14 If you are unable to position both legs correctly, make certain that at least one leg is straight with the foot flexed against the foot piece so that a measurement can be made. The one-leg positioning is the exception rather than the rule and **is only used when children are extremely agitated or uncooperative.**

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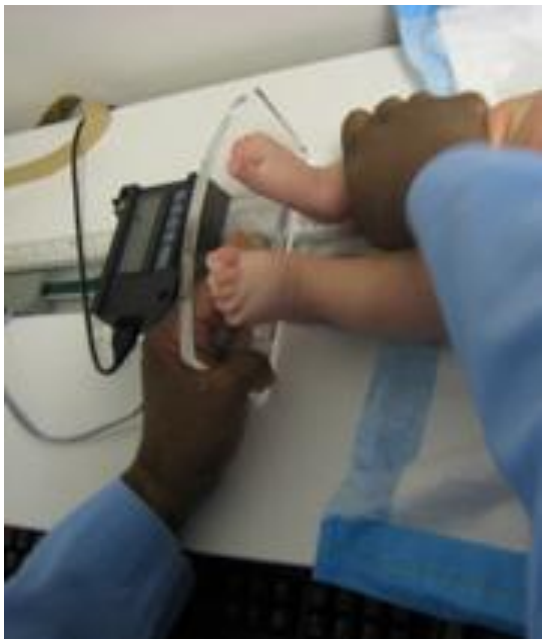

Figure 1. Recumbent length procedure

(Figure adapted from 'WHO MGRS protocol de Onis and Food and Nutrition Bulletin*)*

3.3 Care for measurement equipment

3.3.1 Proper care of the length board is important to ensure that measurements are as accurate as possible.

3.3.2 The equipment must be kept clean and stored at normal indoor temperature, protected from humidity and wetness.

3.3.3 Disposable paper towelling should be used to protect the length board whilst measuring and disposed of after each use.

3.3.4 The length board should be wiped with a detergent and water solution at the end of use.

3.3.5 Any body fluids should be cleaned immediately with a detergent and water solution.

4. TRAINING

4.1 Explain the procedure to a trained staff member.

4.2 Observe a trained staff member performing recumbent length measurements.

4.3 Be observed performing recumbent length measurements, as part of a complete anthropometric profile, following the method outlined in this SOP.

4.4 Trainees must be observed performing measurements on 3 children.

4.5 The trainer must complete and sign the competency checklist (Appendix 1), which can be found at H:\CNRC\Staff Training\Competency Checklists\COMPETENCY CHECKLIST Recumbent Length Measurement

4.6 Accuracy of the trainees measurements must also be assessed.

- **4.6.1** The trainee is to record recumbent length measurements on the Trainee Anthropometric Training Record (Appendix 2), which can be found at H:\CNRC\Staff Training\Trainee Anthropometric Training Record_2012.
- **4.6.2** The trainer is to repeat the measurements, recording their values on the Trainer Anthropometric Training Record (Appendix 3), which can be found at H:\CNRC\Staff Training\Trainer Anthropometric Training Record_2012, and intra-tester and inter-tester Technical Error of Measurement (TEM) assessed.
- **4.6.3** The trainee must achieve an intra-tester TEM of 2.0% and an inter-tester TEM of 2.5%

4.7 Upon satisfactory completion of the competency checklist and anthropometric training record, the trainer must complete the CNRC Internal Training Record (Appendix 4), which can be found at H:\CNRC\Staff Training\CNRC Internal Training Record_2011.

4.8 If the trainee does not satisfactorily complete the training, the procedure must be repeated from step 4.1.

4.9 20 complete anthropometric profiles must be recorded on the Anthropometric Profiles form (Appendix 5), which can be found at H:\CNRC\Staff

Training\Anthropometric Profiles, within the next 6 months of achievement of competency. An intra-tester TEM of 1.5% is required.

4.10 The completed and signed competency checklist, anthropometric training records, internal training record and anthropometric profiles are to be given to the Operations Manager (OM) or Document Controller (DC) for filing in the Staff Training folder.

4.11 Training is to be reviewed every 12 months.

5. GLOSSARY

Operations Manager
Manager for Operation within the CNRC, directly reports to Director of WCHRI.

Document Controller
A person responsible for the distribution and maintenance of SOP's.

6. REFERENCES

1. World Health Organization. Training Course on Child Growth Assessment. Module B "Measuring a Child's Growth. Geneva, WHO, 2008. http:// www.who.int/childgrowth/training/en/ Accessed 10th June 2012.

2. de Onis, M., A. W. Onyango, et al. (2004). "Measurement and standardization protocols for anthropometry used in the construction of a new international growth reference." Food & Nutrition Bulletin **25**(1 Suppl).

7. APPENDIX

Appendix 1. Recumbent length measurement competency checklist
Appendix 2. Trainee anthropometric training record
Appendix 3. Trainer anthropometric training record
Appendix 4. CNRC Internal Training Record
Appendix 5. Anthropometric profiles

Appendix G cont- Head circumference measurement standard operating procedure

Title: Head Circumference Measurement	
Document ID: CNRC_006	**Version : D.V5**
Author(s): Carmel Collins, Jo Collins	
Effective Date:	**Review Before:**
Department/Institution Name: ***Child Nutrition Research Centre***	

Document Revision History			
Version No.	***Reason for Issue / Change***	***Date***	***QA Initial***
1	First Version	21/09/2007	KB
2	Update of SOP format.	12/03/2009	KB
3	*Sub-Sections 3.1.2.1, Figure1 and 4.2*: Added Lufkin tape *Sub-sections 3.2.10, Appendix One:* Included cross-hand technique *Sub-sections 3.2.2 and 3.2.20:* Included guidelines for third measurement. Aligned with ISAK standards.	16/09/2011	JC & CC
4	Added section: 4. Training	31/01/2013	MW
5	Sub-section 3.1.2.2: updated age of use of paper tape from <12 months to <2 years	08/07/2013	MW

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1. INTRODUCTION AND PURPOSE

The objective of this SOP is to detail the procedures required when performing head circumference measurements on study participants involved in clinical trials within the Child Nutrition Research Centre (CNRC) or in collaboration with the CNRC.

Standardisation in the identification of measurement sites, and in measurement techniques are crucial to ensure consistent and accurate results.

2. SCOPE/APPLICABILITY

This SOP applies to all clinical research staff within the CNRC and associated with CNRC clinical trials who are expected to perform or assist with anthropometric measurements. The anthropometry procedures outlined are a guide for measurements performed in relation to CNRC clinical trials, there may be study specific requirements for individual clinical trials which require additional procedures, please refer to your trial protocol.

The procedures comply with the *International standards for anthropometric assessment* [1]

3. PROCEDURE

3.1 Preparation

3.1.1 Follow local area (e.g. WCHN or FMC) guidelines for hand hygiene during procedure.

3.1.2 Assemble equipment required;

3.1.2.1 Lufkin executive thin line (W606PM) tape measure. This is a non-extensible, flexible, <7 mm wide with stub (blank area) of at least 4 cm, steel tape measure. Tape measure wiped with 'Tuffies' between use.

3.1.2.2 For children <2 years of age use a non-extensible single use paper tape calibrated in centimetres with millimetre graduations.

3.1.2.3 Paper or electronic case report form for documenting measurements

3.1.3 Inform the mother and or participant as to what measurement is being taken.

3.1.4 Ensure that the participants' privacy is maintained and that the room is a comfortable temperature.

3.1.5 Child's parent or another adult should be present

3.1.6 Braids or hair ornaments should be removed if they interfere with positioning the tape measure.

3.1.7 Position for measurement will depend upon the age of the child. Head circumference may be measured while the child is lying down, in their mother's arms or standing for an older child.

3.2 Head circumference measurement

General principle for anthropometric measurements

3.2.1 Do a complete anthropometric profile in the order specified by each individual study, and then complete a second anthropometric profile. This may not always be possible, for e.g. with infants, where two consecutive measures are taken.

3.2.2 A third measurement is taken if the first and second measurement differ by >0.5 cm for circumferences.

3.2.3 Be aware of personal space, particularly applies to front of a person, measurements to be taken from side or behind

3.2.4 Work from right side of body

3.2.5 Read measurements at eye level

3.2.6 Do not round up measurements as has not reached that measurement

3.3 Definition

The circumference of the head immediately above the glabella and perpendicular to the long axis of the head.

3.4 Position

3.4.1 Relaxed seated or standing position

3.4.2 Arms hanging by the sides

3.4.3 Head in Frankfort plane

3.5 Method

3.5.1 See cross-hand technique (see appendix 1)

3.5.2 Work from right side of participant

3.5.3 Place the tape around the child's head across the frontal bones just above the eyebrows. The tape should lie above the ears on each side, and over the occipital prominence at the back of the head (Figure 1 and Figure 2).

3.5.4 Hold the tape snugly around the head to compress the hair.

3.5.5 Move the tape up and down over the back of the head to locate the maximal circumference of the head.

3.5.6 Use of the middle fingers at the sides of the head is often necessary to prevent the tape from slipping

3.5.7 Do not include the ears

3.5.8 Record the measurement to the nearest 0.1 cm.

3.5.9 Remove tape

3.5.10 Obtain a second measurement – it is ideal to do one complete anthropometric profile in the order specified by each individual study, and then complete a second anthropometric profile. This may not always be possible, for e.g. with infants, where two consecutive measures are taken).

3.5.11 If the measurements differ by >0.5 cm then repeat a third measurement.

3.6 Care of equipment

3.6.1 Dispose of paper tape measure.

3.6.2 Wipe Lufkin tape measure with site-approved detergent wipes between use.

Figure 1. Head circumference measurement using Lufkin tape measure

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Figure 2. Head circumference measurement in infant using paper tape measure

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4. TRAINING

4.1 Explain the procedure to a trained staff member.

4.2 Observe a trained staff member performing head circumference measurements.

4.3 Be observed performing head circumference measurements, as part of a complete anthropometric profile, following the method outlined in this SOP.

4.4 Trainees must be observed performing measurements on 3 participants.

4.5 The trainer must complete and sign the competency checklist (Appendix 2), which can be found at H:\CNRC\Staff Training\Competency Checklists\COMPETENCY CHECKLIST Head Circumference Measurement

4.6 Accuracy of the trainees measurements must also be assessed.

4.6.1 The trainee is to record head circumference measurements on the Trainee Anthropometric Training Record (Appendix 3), which can be found at H:\CNRC\Staff Training\Trainee Anthropometric Training Record_2012.

4.6.2 The trainer is to repeat the measurements, recording their values on the Trainer Anthropometric Training Record (Appendix 4), which can be found at H:\CNRC\Staff Training\Trainer Anthropometric Training Record_2012, and intra-tester and inter-tester Technical Error of Measurement (TEM) assessed.

4.6.3 The trainee must achieve an intra-tester TEM of 2.0% and an inter-tester TEM of 2.5%

4.7 Upon satisfactory completion of the competency checklist and anthropometric training record, the trainer must complete the CNRC Internal Training Record (Appendix 5), which can be found at H:\CNRC\Staff Training\CNRC Internal Training Record_2011.

4.8 If the trainee does not satisfactorily complete the training, the procedure must be repeated from step 4.1.

4.9 20 complete anthropometric profiles must be recorded on the Anthropometric Profiles form (Appendix 6), which can be found at H:\CNRC\Staff Training\Anthropometric Profiles, within the next 6 months of achievement of competency. An intra-tester TEM of 1.5% is required.

4.10 The completed and signed competency checklist, anthropometric training records, internal training record and anthropometric profiles are to be given to the Operations Manager (OM) or Document Controller (DC) for filing in the Staff Training folder.

4.11 Training is to be reviewed every 12 months.

5. GLOSSARY

Operations Manager
Manager for Operation within the CNRC, directly reports to Director of WCHRI.

Document Controller
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6. REFERENCES

Marfell-Jones M, Olds T, Stewart A, Carter L. *International standards for anthropometric assessment*: International Society for the Advancement of Kinanthropometry: Potchefstroom, South Africa. 2006 WHO MGRS protocol de Onis and Food and Nutrition Bulletin, Vol 25, no 1, page 5

7. APPENDIX

Appendix 1: Cross-hand technique
Appendix 2: Head circumference measurement competency checklist
Appendix 3: Trainee anthropometric training record
Appendix 4: Trainer anthropometric training record
Appendix 5: CNRC internal training record
Appendix 6: Anthropometric profiles

Appendix H - Bioelectrical impedance spectroscopy measurement standard operating procedure

Title: Bioelectrical Impedance Spectroscopy Measurement – ImpSFB7	
Document ID: CNRC_007	**Version : V5**
Author: Carmel Collins	
Effective Date:	**Review Before:**
Department/Institution Name: ***Child Nutrition Research Centre***	

Document Revision History			
Version No.	***Reason for Issue / Change***	***Date***	***QA Initial***
1	First issue	20/02/2012	
2	Revised to include instructions for children as well as infants.		
3	1. Corrected error in naming ChoIR BIS files – changed from 3 year smi61328-03 5 year smi61328-05 CORRECTED to 3 year ch-123456-3 5 year ch-123456-5 2. Addition of check for measurement settings		
4	Minor edits		
5	Added section: 4. Training Removed table from section 3.2		

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1. INTRODUCTION & PURPOSE

Standardisation in measurement techniques is crucial to ensure consistent and accurate results.

The objective of this SOP is to detail the procedures for Bioelectrical Impedance Spectroscopy (BIS) measurements using the ImpSFB7. These procedures should be followed for all assessments associated with CNRC clinical trials.

BIS provides a means of measuring body composition. Body composition gives more information than weight and length alone by giving an estimate of the proportion of weight that is fat in contrast to the "fat-free" body mass which is made up of muscles, bones, and organs.

To do this, BIS estimates total body water and from this, the amount of fat and lean tissue is calculated by the software. BIS measures the opposition of the body to a minute electrical current (200 μA). The current causes no sensation and is less than received when the positive and negative ends of an AA battery are held between the fingers.

Body water is located mainly in the fat-free mass (or lean body tissue); it is highly conductive and provides a low resistance electrical pathway. Fat contains low amounts of fluid and conducting electrolytes and is a poor conductor, providing a high resistance electrical pathway. Total body water will therefore vary according to the relative proportions of fat-free and fat mass; a higher total body water would indicate more fat free mass.

Total body water is a constant fraction of fat-free mass, therefore once total body water is known, the fat-free mass can be calculated. Body fat can then be determined by subtracting the fat-free mass from the body weight. The calculations are performed in the software of the ImpSFB7.

2. SCOPE & APPLICABILITY

This SOP applies to all clinical research staff within the CNRC who performs or assist with BIS measurements. The procedures outlined are a guide for measurements performed in relation to CNRC clinical trials, there may be study specific requirements for individual clinical trials which require additional procedures, please refer to your trial protocol.

3. PROCEDURE

Familiarise with manual ‘Imp SFB7 Instructions For Use’.

Accurate BIS measurement requires

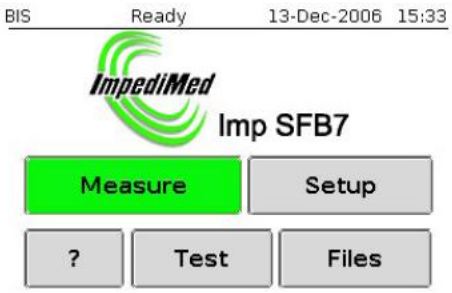
- Training of personnel
- ImpSFB7 calibration and documentation
- Correct position of electrodes
- Correct position of leads
- Correct position of participant
- No skin touching skin

3.1 Preparation

3.1.1 Daily checks and calibration

<u>Before first measurement:</u>

Check date and time displayed is correct. Viewed on top right hand corner of main screen



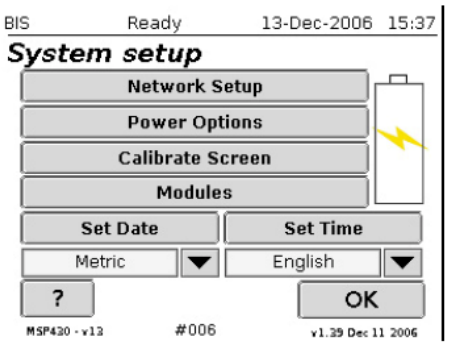
If incorrect date/time go to *Setup/Set date or Set time* to change

Check **set on BIS**. Viewed on top left hand corner

Check battery charge status:

System setup/battery symbol colours green to indicate level of charge.

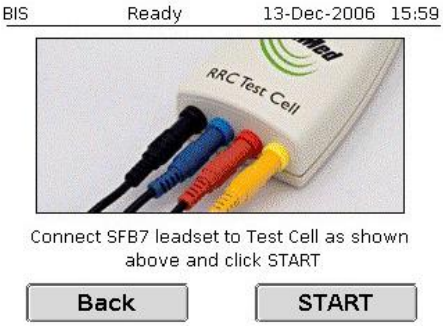
(The indicator light next to the main on/off switch also indicates battery charge status and will change colour (green/orange/red) according to battery status. Please refer to **page 15** of the manual for more information)



Do not take a reading while battery is charging.

Calibration Check instructions

Remove alligator clips and connect the leads to test cell according to the colour-coding.



Press '*Test'* on main screen.

Then press *'start'.*

It will record a pass or failure

If fails, repeat test

If fails again note error message and notify Daniela Calderisi

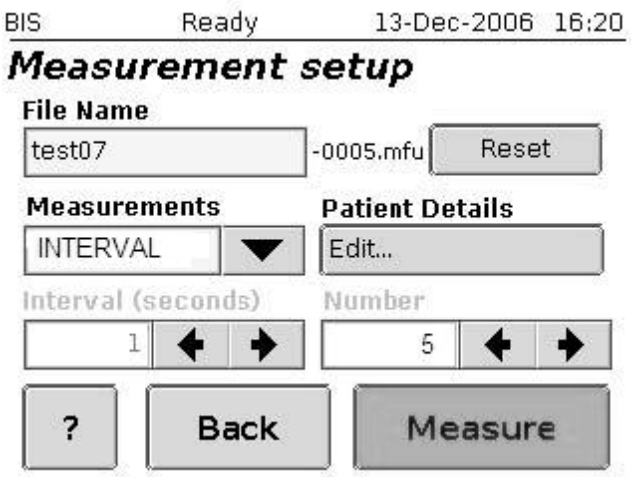
Settings check

Check on correct measurement parameters:

Measurements = Interval

Interval = 1

Number = 5



3.2 Enter participant name

Turn on. Touch '*Measure Setup'*, to open the Measurement Setup screen.

Touch '*File Name'*.

Enter File Name (maximum 12 characters) – see study specific protocols

Do not enter any other participant details, this is done on analysis

3.3 Participant position

Supine position

To prevent short circuit of electrical path:

- Limbs un-crossed and not touching another part of the body OR part of same limb
- Legs completely separated
- No skin touching skin, infant/child or operator/parent

Infants:

- If taking the measurement in the supine position will disturb infant and there is a risk of not getting an accurate reading because infant is unsettled then the reading can be taken in other positions – prone or side lying, but preference is for supine position. Try to extend limbs, i.e. arms not flexed but extended.
- Rolled up nappy or similar can be placed between legs to prevent from touching if needed
- Can restrain limb using gloved hand, or cloth nappy etc to ensure no direct skin contact between operator and infant.
- Cannot be breastfeeding or bottle feeding when measurement taken

Children/Adults: Reading to be taken within 10 minutes of lying in supine position.

3.4 Electrode placement

There are two types of electrodes - single tab and dual tab. The single tab will eventually be phased out by the company.

Minimum distance 2.5 cm between sensing and source electrodes.

If visible matter on skin areas (e.g. powder, cream etc) clean with water – allow to dry thoroughly.

Four electrodes are placed on the LEFT side, two on the foot and two on the hand.

Reseal electrode pouch after opening.

Infant – Single Tab electrodes

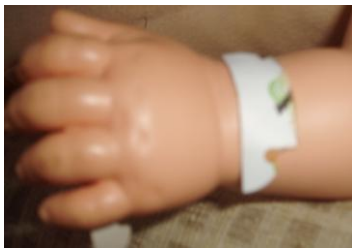
Use single tab electrodes cut in half

Connect alligator clips as follows. **Always double check correct placement of leads**, invalid reading if incorrectly placed.

If electrodes are lifting from the weight/position of the leads, stabilise by wrapping the limb with self-adherent wrap e.g. Coban.

Voltage (sensing) electrode YELLOW.

Wrist - distal edge of the electrode lies along skin crease on the posterior surface of the wrist.

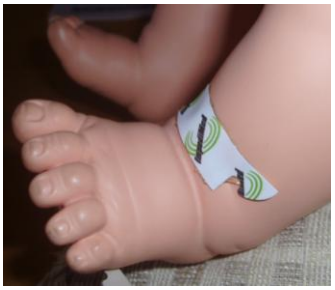


Current (source) electrode RED:

Hand - Placed on the palmar surface of the hand at the distal end of the metacarpals

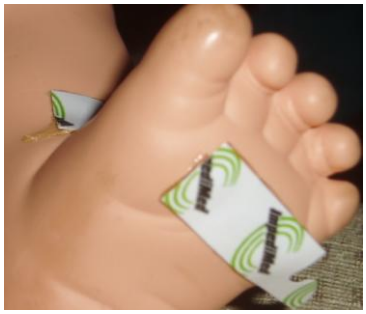
Voltage (sensing) electrode BLUE.

Ankle – distal edge of electrode lies at the level of the medial and lateral malleoli



Current (source) electrode BLACK:

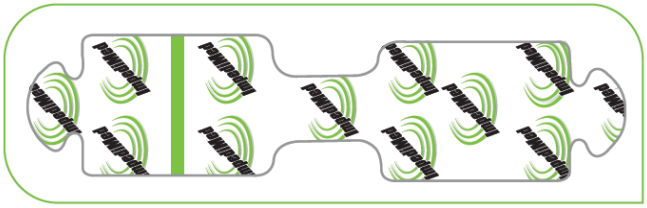
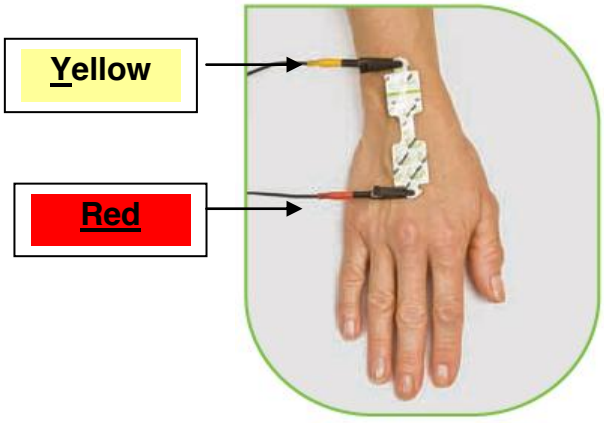
Foot - placed on the plantar surface (sole) of the foot at the distal ends of the metatarsals (Figure 4).

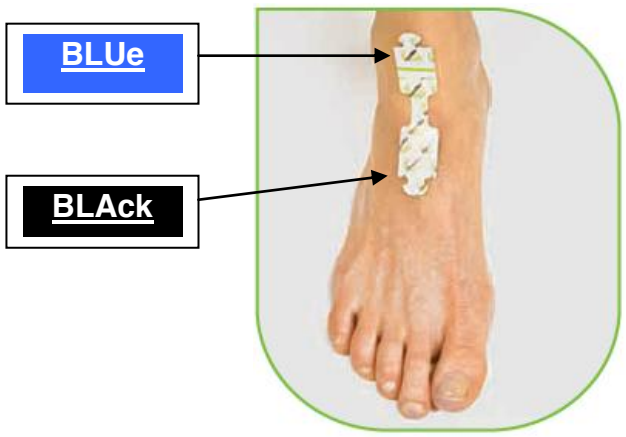


Child/Adult – Dual tab electrodes

Use dual tab electrodes

Connect electrodes and leads as follows. **Always double check correct placement of leads**, invalid reading if incorrectly placed.

Green placement line on dual tab is placed on anatomical land mark.	
Wrist: Place green placement line so as aligns with skin crease on the posterior surface of the wrist. Attach YELLOW lead to wrist Attach RED lead to hand	

Ankle – Place green placement line so as aligns at the level of the medial and lateral malleoli Attach BLUE lead to ankle Attach BLACK lead to foot	

3.5 Taking measurement

Press '*measure'* on the screen, this will take you to another screen,

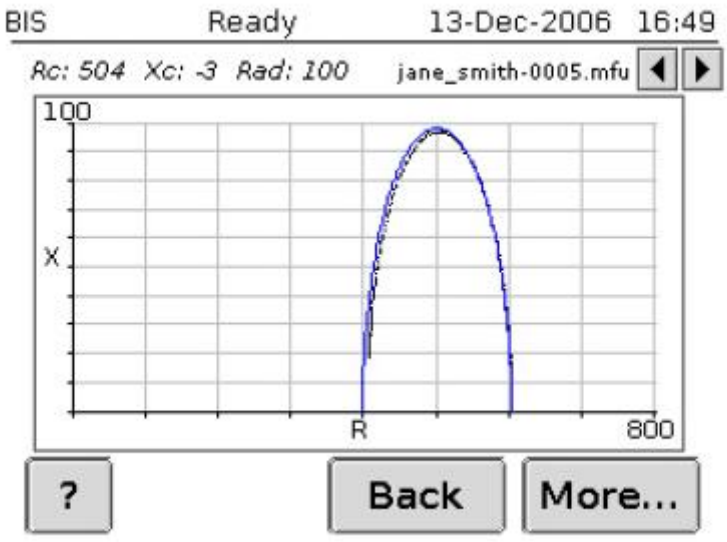
Ensure participant settled and no skin on skin contact

Press '*start'*

Five measurements will be taken at one second intervals.

Observe participant during this time to ensure has not moved and has not touched skin to skin.

Press '*More'* button to inspect Cole-Cole plot (Reactance vs. Resistance). Absence of scattered data points and well fit Cole-Cole plot signifies noise free data and valid measurement.



3.6 Electrode removal

Gently remove electrodes and discard.

Check alligator clips are still in place as they are easily dislodged.

3.7 Documentation

Position of infant, side of body electrodes placed

Document the file name of the FIRST and the LAST measurement taken. A four digit sequential number is automatically added to the end of the patient file name, e.g. smi61328-01-**0001**. The total file name, including this end number are to be documented (do not add file type, i.e. .mfu).

Document the number of measurements taken

Time of last feed (fluid or solids).

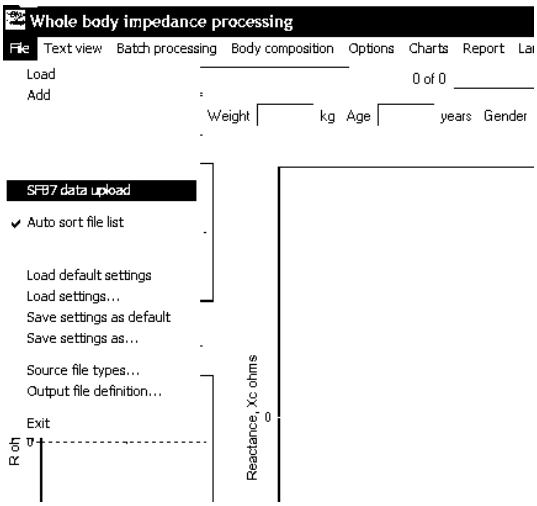
3.8 Data upload

Data is transferred to analysis software via Ethernet.

Connect the red Ethernet lead to SFB7 and to the USB port on your computer.

Click ImpediMed icon on your desktop or access via the 'All Programs' area, when you click on the start menu of computer.

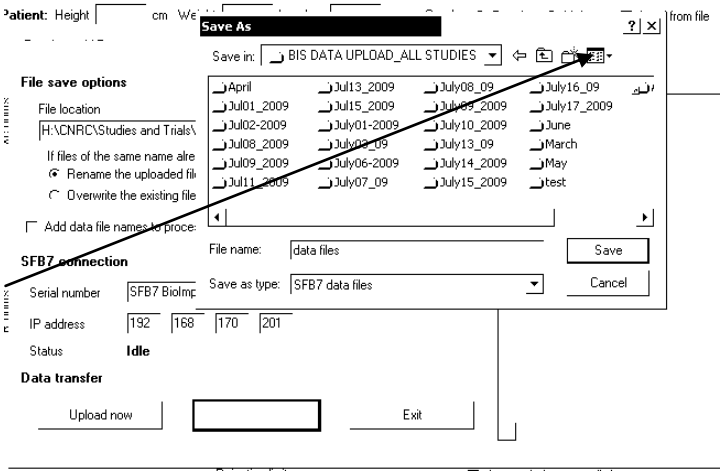
Select File/SFB7 data upload.



To create folder to save files in: click 'Browse', go to:

H:\CNRC\Studies and Trials\BIS DATA UPLOAD_ALL STUDIES

Click on icon to 'create new folder'

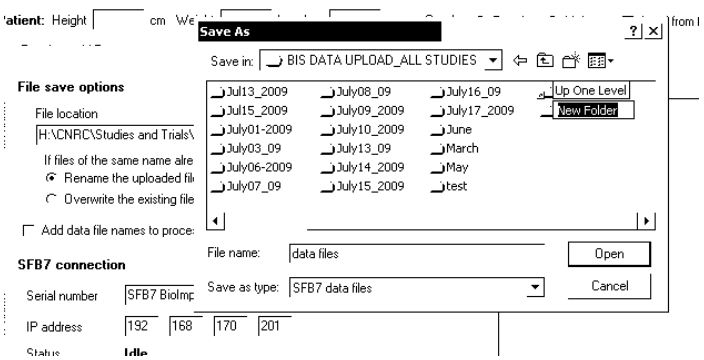


Label folder with the day's date with Month day_Year as follows:

July18_2009

Click 'open'

Click 'save'

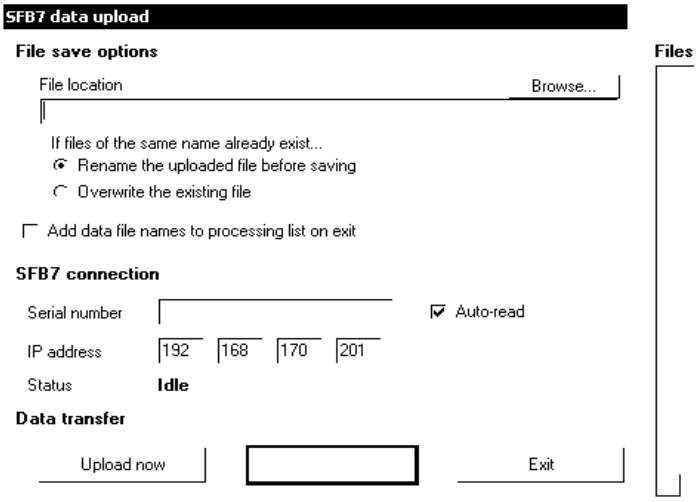


Select *"Rename the uploaded file before saving*"

Select *"Auto-read"*

Click *'Upload now'*

Once uploaded, files are automatically deleted from the Imp SFB7; each file is transferred, confirmed as received and then deleted.



3.9 Quick summary

See appendix one for quick summary

3.10 Care of Equipment

It takes six hours to charge the fully depleted battery. There are 4 to 8 hours of operating time before recharging is needed.

Always use the ImpediMed supplied rubber end of stylus pen to operate the touch screen. Do not use any sharp or metal objects to operate the touch screen. Avoid excessive force as this may damage the screen.

Clean ImpSFB7 with damp cloth. Clean leads with Isowipes (70% alcohol) or tuffies (detergent based).

4. TRAINING

4.1 Familiarise with the manual 'Imp SFB7 Instructions For Use'.

4.2 Explain the procedure to a trained staff member.

4.3 Observe a trained staff member performing BIS measurements.

4.4 Be observed performing BIS measurements following the method outlined in this SOP.

4.5 Trainees must be observed performing measurements on 3 participants.

If will be using on both infants and children, then need to be observed on at least one of each.

4.6 The trainer must complete and sign the competency checklist (Appendix 2), which can be found on the shared drive at: \CNRC\SOP's\Master SOPs\SOP Forms\BIS Measurement Competency Checklist

4.7 Upon satisfactory completion of the competency checklist and anthropometric training record, the trainer must complete the CNRC Internal Training Record (Appendix 3), which can be found on the shared drive at: \CNRC\SOP's\Master SOPs\SOP Forms\CNRC Internal Training Record

4.8 If the trainee does not satisfactorily complete the training, the procedure must be repeated from step 4.2.

4.9 The completed and signed competency checklist and internal training record are to be given to the Operations Manager (OM) or Document Controller (DC) for filing in the Staff Training folder.

4.10 Training is to be reviewed every 12 months.

5. GLOSSARY

Operations Manager
Manager for Operation within the CNRC, directly reports to Director of WCHRI.

Document Controller
A person responsible for the distribution and maintenance of SOP's.

6. REFERENCES

Imp SFB7 Instructions for use

7. APPENDIX

Appendix 1: Quick summary
Appendix 2: BIS measurement competency checklist
Appendix 3: CNRC internal training record

Appendix I - Case report form

POPPET	Study ID: __ __ __ __

POPPET

Providing Optimal Protein for Prems via Enteral Tubes

Does increasing the protein content of human milk fortifier improve growth in preterm infants <33 weeks gestation? A randomised controlled trial.

CASE REPORT FORM

Child Nutrition Research Centre
Women's and Children's Health Research Institute
Women's and Children's Hospital
72 King William Road
North Adelaide, South Australia 5006

Primary contact: Jessica Reid
Pager: 4413
Mobile: 0410 355 129
Phone: 8161 6848
Facsimile: 8161 0267

SECTION 1 Randomisation data

1.1 Randomisation information

Infant UR Number __ __ __ __ __ __ __ __ __ __

Infant Date of Birth __ __ / __ __ / __ __ __ __ *(dd/mm/yyyy)*

Infant time of birth __ __ __ __ *(24 hours)*

Please attach patient label here

Sex
- ☐ Girl
- ☐ Boy

Gestational age
- ☐ <30 weeks gestation *(includes up to 29 weeks and 6 days)*
- ☐ 30 to 32 weeks gestation *(includes 30 weeks to 32 weeks and 6 days)*

Tin colour
- ☐ Purple
- ☐ Yellow
- ☐ Red
- ☐ Green

Study ID __ __ __ __

Randomisation date __ __ / __ __ / __ __ __ __ *(dd/mm/yyyy)*

Randomisation time __ __ __ __ *(24 hours)*

Poppet fortifier start date __ __ / __ __ / __ __ __ __ *(dd/mm/yyyy)*

Poppet fortifier start time __ __ __ __ *(24 hours)*

1.2 Weight, length and head circumference at randomisation

Take 3rd measurement if difference in two weight measurements >5 grams, and in length or HC >0.5 cms. Record two closest measurements

Measure	**Date** __ __ / __ __ / __ __ __ __ *(dd/mm/yyyy)* **Time (24 hours)** __ __ __ __	
	Measurement 1	Measurement 2
Weight	__ __ __ __ g	__ __ __ __ g
Length	__ __ . __ cm	__ __ . __ cm
Head Circumference	__ __ . __ cm	__ __ . __ cm

1.3 Baseline blood sample at randomisation *(need 400 µL green-top EDTA mini-collect tube, no gel separator)*

Date and Time Collected
__ __ / __ __ / __ __ __ __ *(dd/mm/yyyy)* __ __ __ __ *hrs*

1.3.1 Results in database

☐ Yes

☐ No

1.4 Baseline urine sample at randomisation .

Date and Time Collected
__ __ / __ __ / __ __ __ __ *(dd/mm/yyyy)* __ __ __ __ *hrs*

1.4.1 Results in database

☐ Yes

☐ No

1.5 Weight, length and head circumference at study start (within 1-2 days of study fortifier commenced)

Take 3rd measurement if difference in two weight measurements >5 grams, and in length or HC >0.5 cms. Record two closest measurements

Measure	**Date** __ __ / __ __ / __ __ __ __ *(dd/mm/yyyy)* **Time (24 hours)** __ __ __ __	
	Measurement 1	Measurement 2
Weight	__ __ __ __ g	__ __ __ __ g
Length	__ __ · __ cm	__ __ · __ cm
Head Circumference	__ __ · __ cm	__ __ · __ cm

1.6 If commenced on standard fortifier before study start

Must be <4 days before POPPET start.

Date and Time began
__ __ / __ __ / __ __ __ __ *(dd/mm/yyyy)* __ __ __ __ *hrs*

☐ Did not begin standard fortification before POPPET commencement.

Section completed by: (signature)	Date:	Checked by: (initials)	Date:

SECTION 2 CONTACT INFORMATION

2.1 Infant Information

FAMILY NAME: _____ FIRST NAME: _____

2.2 Parental Information

	MOTHER	FATHER
FAMILY NAME		
FIRST NAME		
ADDRESS	_____ _____ State ____ Post Code:__ __ __ __	_____ _____ State _____ Post Code: __ __ __ __
TELEPHONE NUMBERS	Home: _____ Mobile: _____	Home: _____ Mobile: _____

SECTION 3 MEDICAL RECORD DATA COLLECTION

3.1 Mother's Date of Birth: __ __/__ __/__ __ __ __ *(dd/mm/yyyy)*

3.2 Gravida __ __ *(Total number of pregnancies, including this pregnancy)*

3.3 Parity __ __ *(Total number of previous pregnancies resulting in live birth of any gestation or still birth of at least 400g birth weight or 20 weeks gestation).*

3.4 Estimated Due Date (EDD): __ __/__ __/__ __ __ __ *(dd/mm/yyyy)*

3.5 Was there considered to be any clinical or histopathological evidence of chorioamnionitis?

- ☐ Unknown
- ☐ No
- ☐ Yes

3.6 Did the mother smoke <u>during</u> pregnancy?

- ☐ Unknown
- ☐ No
- ☐ Yes, number cigarettes per week ……

3.7 Did the mother drink alcohol <u>during</u> pregnancy?

- ☐ Unknown
- ☐ No
- ☐ Yes, number standard drinks per week ……..

3.8 What is the mother's highest level of secondary school education? *(completed years only)*

Year

- ☐ 8
- ☐ 9
- ☐ 10
- ☐ 11
- ☐ 12
- ☐ Unknown

3.9 What is the mother's highest level of further studies? *(Completed courses only, conducted at registered educational facility, e.g. TAFE, University)*

- ☐ No further study
- ☐ Certificate/Diploma (includes apprenticeships)
- ☐ Degree
- ☐ Higher degree
- ☐ Unknown

3.10 Infant gestational age at birth: __ __ weeks __ days

3.11 Infant received oxygen ☐ Yes ☐ No

3.12 Infant transferred to another hospital

☐ Unknown
☐ No
☐ Yes (complete next table)

Hospital	Date transferred
	__ __ / __ __ / __ __ __ __
	__ __ / __ __ / __ __ __ __
	__ __ / __ __ / __ __ __ __
	__ __ / __ __ / __ __ __ __

3.13 Total number days any parenteral nutrition: __ __ __ days *(during study, include part days as 1 to EDD or discharge)*

3.14 Total number days any intravenous lipids: __ __ __ days *(during study, include part days as 1 to EDD or discharge)*

3.15 Enrolled in other studies *(Is the infant enrolled in any other studies)*

☐ Unknown
☐ No
☐ Yes *(complete next table)*

Study	Chief Investigator, Institution	Study ID

3.16 Infant death *(death occurring prior to discharge from hospital)*

☐ No – survived to discharge home
☐ Yes *(CI to complete 4.18.3, 4.18.4)*

3.16.1 Date of death __ __/__ __/__ __ __ __

3.16.2 Copy of post mortem report

☐ No post mortem performed
☐ Yes, a post mortem was performed and copy of report obtained

3.16.3 Notification of death form

☐ No
☐ Yes, completed

3.16.4 Ethics committee notified

☐ No
☐ Yes, completed

SECTION 4 DAILY ASSESSMENT (as many pages as needed)

DATE	/ /	/ /	/ /	/ /	/ /	/ /	/ /
Study day	1	2	3	4	5	6	7
Daily weight (gms)							
Length Measure (cm)							
Head Circum. Measure (cm)							
Recorded / prescribed human milk intake (mls)							
Recorded number of breast feeds							
Poppet fortifier intake							
No of times study fortifier given/number of feeds	/	/	/	/	/	/	/
Recorded formula intake(mls)							
Feeds of >50% formula							
Name & caloric strength							
Feeds held or not given	Y N	Y N	Y N	Y N	Y N	Y N	Y N
Protifar (g/kg)							
MCT (ml/day)							
Polyjoule							
Other supplement							

Section completed by: (signature)	Date:	Checked by: (initials)	Date:

SECTION 5 WEEKLY STUDY PROCESSES

5.1 Blood samples *(500 µL in green EDTA-top mini-collect tube, no gel separator)*

Entered into database Y/N. Acid/base includes pH, pco2, pHCO-3, base excess.

	Urea	Albumin	Creatinine	Electrolytes	Acid/base	aa profile
Week 1. __/__/____ Time __ __.__ __						
Week 2. __/__/____ Time __ __.__ __						
Week 3. __/__/____ Time __ __.__ __						
Week 4. __/__/____ Time __ __.__ __						
Week 5. __/__/____ Time __ __.__ __						
Week 6. __/__/____ Time __ __.__ __						
Week 7. __/__/____ Time __ __.__ __						
Week 8. __/__/____ Time __ __.__ __						
Week 9. __/__/____ Time __ __.__ __						
Week 10. __/__/____ Time __ __.__ __						
Week 11. __/__/____ Time __ __.__ __						
Week 12. __/__/____ Time __ __.__ __						
Week 13. __/__/___ Time __ __.__ ___						
Week 14. __/__/____ Time __ __.__ __						
Week 15. __/__/____ Time __ __.__ __						
Week 16. __/__/____ Time __ __.__ __						

SECTION 6 BREAST MILK SAMPLE

Collect 8 mls expressed breast milk.

If not collected give reason eg inadequate supply.

Collection date refers to date milk is thawed to be given to the baby and collected for a sample

Expression date refers to date milk expressed by mother.

Wk 1.Collection__/__/____	☐ Yes
Expression __/__/____	☐ No_____
Wk 2.Collection__/__/____	☐ Yes
Expression __/__/____	☐ No_____
Wk 3.Collection__/__/____	☐ Yes
Expression __/__/____	☐ No_____
Wk 4.Collection__/__/____	☐ Yes
Expression __/__/____	☐ No_____
Wk 5.Collection__/__/____	☐ Yes
Expression __/__/____	☐ No_____
Wk 6.Collection__/__/____	☐ Yes
Expression __/__/____	☐ No_____
Wk 7.Collection__/__/____	☐ Yes
Expression __/__/____	☐ No_____
Wk 8.Collection__/__/____	☐ Yes
Expression __/__/____	☐ No_____
Wk 9.Collection__/__/____	☐ Yes
Expression __/__/____	☐ No_____
Wk 10.Collection__/__/____	☐ Yes
Expression __/__/____	☐ No_____
Wk 11.Collection__/__/____	☐ Yes
Expression __/__/____	☐ No_____
Wk 12.Collection__/__/____	☐ Yes
Expression __/__/____	☐ No_____
Wk 13.Collection__/__/____	☐ Yes
Expression __/__/____	☐ No_____
Wk 14.Collection__/__/____	☐ Yes
Expression __/__/____	☐ No_____

Section completed by: (signature)	Date:	Checked by: (initials)	Date:

SECTION 7 Body Composition

	Time of last feed	Electrodes attached	First measurement	Last measurement
Week 1. __/__/____ Time __ __.__ __		L / R		
Week 2. __/__/____ Time __ __.__ __		L / R		
Week 3. __/__/____ Time __ __.__ __		L / R		
Week 4. __/__/____ Time __ __.__ __		L / R		
Week 5. __/__/____ Time __ __.__ __		L / R		
Week 6. __/__/____ Time __ __.__ __		L / R		
Week 7. __/__/____ Time __ __.__ __		L / R		
Week 8. __/__/____ Time __ __.__ __		L / R		
Week 9. __/__/___ Time __ __.__ __		L / R		
Week 10. __/__/____ Time __ __.__ __		L / R		
Week 11. __/__/____ Time __ __.__ __		L / R		
Week 12. __/__/____ Time __ __.__ __		L / R		
Week 13. __/__/____ Time __ __.__ __		L / R		
Week 14. __/__/____ Time __ __.__ __		L / R		

Section completed by: (signature)	Date:	Checked by: (initials)	Date:

SECTION 8 BUN and Metabolic Acidosis

8.1 BUN >8mmol/L and/or a metabolic acidosis (base excess <-6) *(See Appendix, page 24)*

☐ No

☐ Yes go to 8.2

8.2

Episode	Date	BUN	Base Excess	High after 48 hours
1	__ __ / __ __ / __ __ __ __			□ Yes □ No
2	__ __ / __ __ / __ __ __ __			□ Yes □ No
3	__ __ / __ __ / __ __ __ __			□ Yes □ No
4	__ __ / __ __ / __ __ __ __			□ Yes □ No
5	__ __ / __ __ / __ __ __ __			□ Yes □ No
6	__ __ / __ __ / __ __ __ __			□ Yes □ No
7	__ __ / __ __ / __ __ __ __			□ Yes □ No
8	__ __ / __ __ / __ __ __ __			□ Yes □ No

8.3

Episode	Date	BUN	Base excess
1	__ __ / __ __ / __ __ __ __		

8.4 Was the infant switched to standard clinical fortifier (1g protein/100 ml) for 48 hours?

☐ No

☐ Yes __ __/__ __/__ __ __ __ (dd/mm/yyyy) go to 8.41

8.5 Did the infant return to study fortifier?

☐ No BUN remained > 8 mmo/L

☐ No Clinical concern _____

☐ No Metabolic Acidosis (<-6)

☐ Yes __ __/__ __/__ __ __ __ (dd/mm/yyyy)

8.6

Episode	Date	BUN	Base excess
2	__ __ / __ __ / __ __ __ __		

8.7 Was the infant switched to standard clinical fortifier (1g protein/100 ml) for 48 hours?

☐ No

☐ Yes __ __/__ __/__ __ __ __ (dd/mm/yyyy) go to 8.41

8.8 Did the infant return to study fortifier?

☐ No BUN remained > 8 mmo/L

☐ No Clinical concern _____

☐ No Metabolic Acidosis (<-6)

☐ Yes __ __/__ __/__ __ __ __ (dd/mm/yyyy)

8.9

Episode	Date	BUN	Base excess
3	__ __ / __ __ / __ __ __ __		

8.10 Was the infant switched to standard clinical fortifier (1g protein/100 ml) for 48 hours?

☐ No

☐ Yes __ __/__ __/__ __ __ __
(dd/mm/yyyy) go to 8.41

8.11 Did the infant return to study fortifier?

☐ No BUN remained > 8 mmo/L
☐ No Clinical concern _____
☐ No Metabolic Acidosis (<-6)
☐ Yes __ __/__ __/__ __ __ __ (dd/mm/yyyy)

8.12 How many days did the infant not have study fortifier because of BUN/Metabolic Acidosis?

___ ___

8.13 Was Protifar prescribed to the infant?

☐ No
☐ Yes

Episode	Start Date	BUN	End Date	BUN
1	__ __ / __ __ / __ __ __ __		__ __ / __ __ / __ __ __ __	
2	__ __ / __ __ / __ __ __ __		__ __ / __ __ / __ __ __ __	
3	__ __ / __ __ / __ __ __ __		__ __ / __ __ / __ __ __ __	
4	__ __ / __ __ / __ __ __ __		__ __ / __ __ / __ __ __ __	

8.14 How many total days was the infant prescribed Protifar?

___ ___

SECTION 9 CLINICAL INFORMATION (maternal and neonatal from CIS)

9.1 Mother's UR NUMBER: __ __ __ __ __ __ __

9.2 Maternal age in completed years at time of baby's birth: __ __

9.3 Ethnicity of Mother: *(ethnic origin of the mother of baby, as identified by the mother)*

0 Unknown
1 Aboriginal or Torres Strait Islander – by descent who identifies as an Aboriginal or TI and is accepted as such by the community with which she is associated
2 Asian (from countries of Asia including Fijian Indian)
3 Caucasian (includes Arabic, European, Russian, Middle Eastern)
4 Other (includes Black African, Inuit, African American, American Indian, Melanesian)
specify _____
5 Pacific Islander
6 Maori

9.4 Assisted conception

0 Unknown
1 None used for this pregnancy
2 Hyperovulation – any hormone used to stimulate ovulation
3 IVF/GIFT etc
4 Other - infertility treatment not mentioned above, including artificial insemination

9.5 Previous preterm births *(>20 completed weeks, <37 completed weeks gestation)*

☐ Unknown
☐ No previous preterm birth
☐ Yes, there was a previous preterm birth

9.6 Previous perinatal death *(>20 completed weeks or >400 grams and died during first 28 days of life)*

☐ Unknown
☐ No previous perinatal death
☐ Yes, has had a previous perinatal death

9.7 Presenting antenatal problem *(See Appendix, page 40, for definitions)*

0 Unknown, information not available
1 Preterm pre-labour rupture of membranes
2 Preterm labour
3 Hypertension in pregnancy
4 Antepartum haemorrhage
5 Suspected intrauterine growth restriction
6 Fetal distress
7 Other (please specify) ……
8 None − No presenting problem. Baby must be born at term.

9 Antenatal diagnosis of fetal malformation − fetal malformation diagnosed prior to birth by any method.

9.8 Did the mother have antenatal corticosteroids during this pregnancy?

0 Unknown
1 None – steroids not given
2 < 24 hours - first dose given <24 hours prior to this baby's birth
3 Complete – more than 1 dose of steroids given, and 1 dose at >24 hours and <8 days before birth
4 Given at > 7 days before baby's birth

Note: If two courses given and one fulfils the 'complete' criteria, use 'complete'. If the time of doses given is not available, but two doses are known to have been given appropriately, also use 'complete'.

9.9 Mode of Birth/Delivery:

0 Unknown
1 Vaginal – vaginal birth, includes breech
2 Instrument – vaginal birth using instrument; forceps, rotations, vacuum extraction.
3 Caesarean section in labour – caesarean performed after the commencement of labour.
4 Caesarean section, no labour – caesarean section performed prior to labour commencing.

9.10 Plurality *(See Appendix, page 24, for definitions)*

1 Singleton
2 Twins
3 Triplets

9.11 Birth Order

1 Singleton
2 First of a multiple birth
3 Second of a multiple birth
4 Third of a multiple birth

9.12 Apgar score.

1 minute __ __
5 minutes __ __

9.13 Birth weight: __ __ __ __ grams

9.14 Birth length: __ __ · __ cm

9.15 Birth head circumference: __ __ · __ cm

9.16 Date of final discharge home __ __/__ __/__ __ __ __ *(dd/mm/yyyy)*

9.17 Discharge weight: __ __ __ __ grams

9.18 Discharge length: __ __ · __ cm

9.19 Discharge head circumference: __ __ · __ cm

9.20 Main respiratory diagnosis *(See Appendix, page 23, for definitions):*

0 Unknown
1 Normal.
2 Non specific.
3 Hyaline membrane disease.
4 Meconium aspiration.
5 Pneumonia
6 Persistent pulmonary hypertension.
7 Superseded
8 Apnoea
9 Congenital malformation.
10 Other.
11 Peri surgical.
12 Newborn encephalopathy.

9.21 Inpatient date of final added oxygen (O2) therapy: __ __/__ __/__ __ __ __ *(dd/mm/yyyy)*

9.22 Home oxygen ☐ **Yes** ☐ **No**

9.23 Hours of intermittent positive pressure (IPPV) __ __ __ __ IPPV hours

Note: The hours of all forms of assisted ventilation via an endotracheal tube are summed. The usual rounding up applies, e.g.. 1hour 30 minutes is 2 hours. For prolonged use of this therapy, i.e. more than 72 hours, round up to the nearest day (24 hours).

9.24 High Frequency Ventilation (HFOV): ☐ **Yes** ☐ **No**

9.25 Hours of continuous positive airways pressure (CPAP) __ __ __ __ CPAP hours

Note: The total number of hours of CPAP via any route, and of nasopharyngeal ventilation, are summed. The usual rounding up applies, e.g.. 1hour 30 minutes is 2 hours. For prolonged use of this therapy, i.e. more than 72 hours, round up to the nearest day (24 hours).

9.26 Nitric Oxide: ☐ **Yes** ☐ **No**

9.27 Maximum Grade of Intraventricular Haemorrhage (IVH) *(Worst level of IVH seen on either side by ultrasound):*

0 None – no IVH.
1 Grade 1 - Subependymal germinal matrix IVH.
2 Grade 2 - IVH with no ventricular distension.
3 Grade 3 - The ventricle is distended with blood.
4 Grade 4 - Intraparynchymal haemorrhage.
5 Not examined.

9.28 Cerebral cystic formations *(changes in brain parenchyma seen at the scan closest to six weeks of age)*

0 Unknown
1 No cysts – none seen on ultrasound
2 Porencephalic cysts(s) – parenchymal lesions corresponding to grade IV IVH
3 Periventricular leukomalacia – ischaemic brain injury affecting periventricular white matter in the boundary zones supplied by terminal braches of both cetripetal and cetrifugal arteries
4 Encephaloclastic porencephaly – relatively late development on cerebral scan of extensive dense, cystic lesions involving the periphery of the brain

9.29 Retinopathy of prematurity *(Worst stage of ROP in either eye prior to going home)*

0 None seen – no changes seen
1 Stage I
2 Stage II
3 Stage III
4 Stage IV
5 Not examined – no eye examination performed

9.30 Proven Necrotising Enterocolitis *(See Appendix, 24, for definitions)*

99 Unknown
0 No necrotising enterocolitis proven.
1 Yes, necrotising enterocolitis proven (complete next table).

Episode	Date
1	__ __ / __ __ / __ __ __ __
2	__ __ / __ __ / __ __ __ __
3	__ __ / __ __ / __ __ __ __

9.31 Surgery *(Has the infant undergone any surgery)*

☐ Unknown
☐ No
☐ Yes (complete next table)

Episode	Surgical procedure	Date
1		__ __ / __ __ / __ __ __ __
2		__ __ / __ __ / __ __ __ __
3		__ __ / __ __ / __ __ __ __
4		__ __ / __ __ / __ __ __ __

9.32 Postnatal steroids *(Has the infant had any corticosteroid use)*

☐ Unknown
☐ No
☐ Yes (complete next table)

Corticosteroid	Route *(IV, Oral, topical)*	Start date	Stop date
		__ __ / __ __ / __ __ __ __	__ __ / __ __ / __ __ __ __
		__ __ / __ __ / __ __ __ __	__ __ / __ __ / __ __ __ __
		__ __ / __ __ / __ __ __ __	__ __ / __ __ / __ __ __ __
		__ __ / __ __ / __ __ __ __	__ __ / __ __ / __ __ __ __

9.33 Exogenous surfactant

0 Unknown
1 None – no exogenous surfactant ever given.
2 Exosurf.
3 Survanta.
4 Both – any combination of surfactant.
5 Other – use of other surfactant
6 Curosurf
7 Curosurf and survanta

9.34 Early onset sepsis

The presence of at least one episode of systemic sepsis with initial symptoms occurring prior to 48 hours after birth.

☐ Unknown
☐ No
☐ Yes (complete next table)

Episode	Organism(s)
1	
2	
3	
4	

9.35 Late onset sepsis

The presence of at least one episode of blood or CSF infection with initial symptoms occurring from 48 hours after birth.

☐ Unknown
☐ No
☐ Yes (complete next table)

Episode	Organism(s)
1	
2	
3	
4	

9.36 Full enteral feeds (≥ 150 mls/kg/day) – date first reached:

__ __/__ __/__ __ __ __ (dd/mm/yyyy)

9.37 Chronic Lung Disease *(See Appendix, page 24)*

☐ Unknown
☐ No chronic lung disease.
☐ Yes, chronic lung disease.

9.38 Packed Red Blood Cell Transfusion

☐ Unknown
☐ No.
☐ Yes

Episode	Date	Fasting
1	__ __ / __ __ / __ __ __ __	Y / N
2	__ __ / __ __ / __ __ __ __	Y / N
3	__ __ / __ __ / __ __ __ __	Y / N
4	__ __ / __ __ / __ __ __ __	Y / N

Section completed by: (signature)	Date:	Checked by: (initials)	Date:

SECTION 10 APPENDIX

10.1 Primary reason for pre-term birth

The antenatal complication that the mother presented with in this pregnancy, that started the train of events that lead to this baby's birth. Coding of the primary cause of preterm birth is as per the ANZNN[1] criteria, which have been reproduced below.

Preterm pre-labour rupture of membranes; confirmed spontaneous rupture of membranes occurring prior to the onset of labour, and before 37 completed weeks gestation. Rupture of the membranes is defined as the obvious gush or clear amniotic fluid from the vagina, or (if fluid is available) by differentiation with urine and vaginal secretions.

Preterm labour is defined as the presence of regular painful contractions leading to progressive effacement and dilatation of the cervix, eventually leading to the birth of the baby, commencing before 37 completed weeks gestation.

Hypertension in pregnancy is defined as either; A systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg, or a rise in systolic blood pressure ≥ 25 mmHg and/or a rise in diastolic blood pressure ≥ 15 mmHg from blood pressure reading before conception or in the first trimester (confirmed by two readings six hours apart).

Antepartum haemorrhage: Significant haemorrhage in the time from 20 weeks gestation to the end of the second stage of labour. This excludes a “show”.

Suspected intra-uterine growth restriction is the failure of fetus to reach its genetically predetermined full growth potential due to intrinsic or extrinsic factors, based on more than one obstetric ultrasound.

Fetal distress is any distress of this fetus leading to intervention by the obstetric team.

Other – Other significant antenatal complication, not specified

None − No presenting problem. Baby must be born at term.

Antenatal diagnosis of fetal malformation − fetal malformation diagnosed prior to birth by

any method. This prenatal diagnosis may or may not be confirmed after birth.

In cases of babies from multiple births, complication relates to this baby only. Multiple pregnancy is not a presenting antenatal problem; it is coded under ‘plurality’.

10.2 Main respiratory diagnosis.

Code	Main indication for respiratory support
1	Normal – no respiratory support
2	Non specific – any non specific respiratory distress (RD) in an infant requiring respiratory support
3	Hyaline membrane disease – increasing RD or oxygen (O2) requirements, or the need for ventilator support from the first 6 hours of life with a chest x-ray showing generalized reticulogranular pattern, plus or minus bronchogram
4	Meconium aspiration – RD presenting from immediately after birth to 12 hours of age. Hypoxia, tachypnoea, gasping respirations and often signs of underlying asphyxia. Chest x-ray shows over-expansion of lungs with widespread coarse, fluffy infiltrates.
5	Pneumonia – RD with proven or suspected infection (toxic blood count) and chest x-ray showing persisting opacities.
6	Persistent pulmonary hypertension – echocardiac (shunting or clinical evidence – O2 needed unexplained by chest x-ray or loud P2, or differential pre/post ductal TCPO2).
7	Superseded
8	Apnoea - recurrent pauses in breathing of more than 20 seconds, or for less than 20 seconds and associated with bradycardia or desaturation requiring intervention.
9	Congenital malformation – malformation is the primary reason for RD e.g. diaphragmatic hernia.
10	Other – unspecified other RD.
11	Peri surgical – no RD, support given for surgical intervention.
12	Newborn encephalopathy – a syndrome of disturbed neurological function in an infant with difficulties initiating or maintaining respiration, depression of tone reflexes or consciousness and often with seizures.

10.3 Proven necrotising enterocolitis

1. Diagnosis at surgery or post mortem, or
2. Radiological diagnosis, a clinical history plus
    a. pneumatosis intestinalis, or
    b. portal vein gas, or
    c. a persistent dilated loop on serial X-rays, or
3. Clinical diagnosis, a clinical history plus abdominal wall cellulitis and palpable abdominal mass.

10.4 Plurality of pregnancy

Plurality of a pregnancy is determined by the number of live births or by the number of fetuses that remain in utero at 20 weeks gestation and that are subsequently born separately. In multiple pregnancies or, if gestational age is unknown, only live births of any birth weight or gestational age, or fetuses weighing 400 g or more are taken into account in determining plurality. Fetuses aborted before 20 completed weeks or fetuses compressed in the placenta at 20 or more weeks are excluded.

10.5 Chronic lung disease

Derived data – Baby received respiratory support for a chronic pulmonary disorder at 36 weeks post menstrual age: Date of final added oxygen therapy must be > Date of birth or {[(Hours of IPPV + Hours of CPAP)/168] + Gestational age} > 35.9. Main respiratory diagnosis must be > 1.

10.6 BUN and Metabolic Acidosis

After discussion with neonatologists and for the purposes of this study population uraemia is defined as BUN levels above 8.0 mmol/L.

Infants on full enteral feeds who develop a BUN >8.0 mmol/L and/or a metabolic acidosis (base excess <-6) will have the BUN and blood gas analysis repeated within 48 hours and attending clinician notified.

If BUN remains elevated and

1) is associated with acidosis then the study fortifier will be discontinued and infant commenced on S26-SMA fortifier (1g protein/100 ml) for 48 hours and managed as clinically indicated. If acidosis is corrected, the infant may continue in the study and blood tests will monitored twice weekly for one week.

2) is not associated with other abnormal blood tests the infant may remain in the study with BUN and chemistries monitored twice weekly until returns to normal.

Infants who have poor weight gain (defined as <15 g/kg/day over the preceding 7 day period) associated with a BUN <2 mmol/L once full enteral feeds have been reached will be assessed by the attending neonatologist. Feeds will be increased to 170 to 180 mls/kg/day. If weight gain does not improve then additional protein supplements (Protifar) may be added at the discretion of the attending neonatologist. Once a weight gain of 15g/kg/day is achieved and BUN levels ≥2 mmol/L additional protein will be ceased

Appendix J - Data analysis plan

1. PREFACE

This Statistical Analysis Plan (SAP) describes the planned analyses and reporting for the Poppet Study. This study is being completed to assess the safety and efficacy of supplementing expressed breast milk feeds and direct breast feeds with increased protein human milk fortifier in premature infants.

The following documents were reviewed in preparation of this document:

- Poppet Study Protocol Version 3, 23 January 2012
- Poppet Case Report Form Version 1, 7 October 2011.
- Poppet Note to file, 2 May 2012.

2. STUDY OBJECTIVES AND OUTCOMES

2.1. Study Objectives

POPPET stands for Providing Optimal Protein for Prems via Enteral Tubes.

The primary objective of POPPET was to determine the effect of supplementing expressed breast milk (EBM) and direct breast feeds with an increased protein content human milk fortifier on the rate of weight gain.

2.2. Outcome Variables

The primary outcome variable is rate of weight gain.

The primary outcome and all secondary outcomes are defined in detail in section 0 of the SAP.

Study start was randomisation (irrespective of when fortifier was begun). Study end was defined as day of removal of naso-gastric tube.

3. STUDY METHODS

3.1. Study design

Parallel group single centre randomised controlled trial.

3.2. Participants

Inclusion criteria:

- Infants born <33 weeks gestation
- Infants born to women who were planning to breast feed

Exclusion criteria:

- Infants who were likely to transfer to rural hospitals where follow-up was problematic
- Infants born with chromosomal/congenital abnormality
- Infants born to women who could not offer informed consent

3.3. Method of Treatment Assignment and Randomisation

Sequence generation: The randomisation schedule was devised using a computer generated randomisation schedule.

Stratification occurred for gestational age (<30 weeks and 30-32 weeks) and sex as defined in Table 1. Multiple births were randomised individually with the first born infant randomised first.

Table 1. Stratification variables

Stratification Variable	Categories
Gestational age	<30 weeks 30 – 32 weeks
Infant sex	Male Female

Allocation concealment: Randomisations were allocated by telephone by research assistants who were not otherwise connected to the Poppet Study. Infants were randomised to receive high protein human milk fortifier or standard protein human milk fortifier.

Implementation: The random allocation sequence was generated by statistician not involved in the conduct of the study. Interventions were assigned by telephoning research assistants who were not involved in the conduct of the study.

3.4. Intervention

Intervention: High protein human milk fortifier

Control: Standard protein human milk fortifier

3.5. Blinding

The study products (high protein-Protifar, standard protein-PolyJoule) were assigned two different coloured tins each resulting in four different coloured tins. The POPPET solution was made up by Infant Nutrition Attendants in the Diet Kitchen separate from the nursery. The Infant Nutrition Attendants were not involved in the care of the infants. Study tins were locked in a cupboard when not in use. Infant caregivers did not have access to this room.

Parents, caregivers, research staff and statistical staff were blinded to treatment allocation.

4. SEQUENCE OF PLANNED ANALYSES

4.1. Interim Analyses

There are no planned interim analyses for this study.

4.2. Final Analyses and Reporting

Once the study has been completed and all data have been entered, a blinded review of the data will be conducted and final changes will be made to this SAP. No statistical analyses will be performed until the final version of this SAP has been approved.

Blinded treatment codes will be included in the database and analysis of the primary outcomes will be performed blinded to treatment group. Analysis of secondary outcomes directly related to the primary outcomes (i.e. those listed in Sections 9.2 and 9.3) will also be performed blinded to treatment group. The blinding will be broken following the analysis of these outcomes. Analysis of all other secondary outcomes will be performed after the blinding has been broken. Results of the statistical analyses will be made available to the Chief Investigators.

Any post-hoc, exploratory analyses which were not identified in this SAP but are completed to support planned study analyses will be clearly identified in the final report.

5. SAMPLE SIZE DETERMINATION

5.1. Rate of weight gain

To demonstrate an increase in rate of weight gain of 3.31 grams per day with 80% power (alpha=0.05) we will require a sample size of 60, 30 per group. This difference in rate of weight gain is considered clinically relevant and the sample size achievable within the time frame of a PhD.

6. GENERAL ISSUES

6.1. Analysis software

All analyses will be performed using SAS® version 9.3 or later.

6.2. Analysis Approach

Randomised participants will be analysed according to the treatment they were randomised to receive.

6.3. Methods for withdrawals, missing data, outliers.

Data collected on participants up until the point of withdrawal will be included in the analysis. Data after the point of withdrawal will be collected and used where permission has be obtained.

Missing data will not be imputed.

Outliers will be queried during data collection and the statistical analysis. Unless confirmed as a data entry error, outliers will not be excluded from the primary analysis.

6.4. Protocol Violations and Deviations

No participants will be excluded from the analyses due to protocol violations or deviations.

6.5. Data transformations

No data transformations are planned. The statistical analyses detailed in Section 9 are based on assumptions about the distribution of the outcomes. Should these assumptions turn out to be invalid, appropriate data transformations may be required.

6.6. Potential Confounders

In order to address each hypothesis, both unadjusted and adjusted analyses will be performed. The adjusted results will be used to draw conclusions about the effect of treatment with unadjusted analyses performed for completeness and to potentially confirm the results of the adjusted analyses.

Since gestational age and sex were used as stratification variables in the randomisation process, all analyses will be adjusted for gestational age and sex. These will be defined as in Section 0.

Sex will not be included as a confounder for those outcomes already standardised by sex.

Any deviation from the planned adjustment for potential confounders will be clearly identified in the final report.

6.7. Planned per protocol analysis

A per protocol analysis of primary and secondary outcomes will be undertaken and will exclude data from infants where Poppet study solution was given with ≤70% of total enteral intake over the study period.

6.8. Multiple Comparisons and Multiplicity

Multiple hypothesis tests will need to be performed to assess the effectiveness of higher protein due to multiple primary and secondary outcomes, unadjusted and adjusted analyses and planned treatment by covariate interactions.

There is only one primary outcome.

No adjustment will be made for the number of secondary outcome analyses performed or any planned per protocol analyses as these analyses are of less importance and less emphasis will be placed on the results.

Since conclusions will be drawn based on the adjusted results no adjustment will be made for the fact that both adjusted and unadjusted analyses are being performed on for each outcome.

7. DESCRIPTIVE STATISTICS

7.1. Screening Population

Descriptive information relating to the screened population (i.e. all participants screened for potential inclusion in the study) will be presented, including consent rates and reasons for non-consent as specified by the CONSORT statement.

7.1. Disposition of participants and withdrawals

Information will be presented by treatment group (where appropriate) on:

- The number of infants screened
- The number of infants who were ineligible by reason
- The number of infants who were eligible
- The number of eligible infants who did not have consent provided by reason.
- The number of eligible infants who had informed consent given and were randomised
- The number of infants who did not complete the study by reason
- The number of infants who completed the primary outcome assessment (to removal of feeding tube)
- The number of infants who completed any of the outcomes to study end

7.2. Randomisation and Selection Errors

Information will be presented by treatment group on:

- The number of infants randomised in the wrong stratum by reason
- The number of infants given the wrong treatment
- The number of infants discovered to be ineligible after randomisation by reason

7.3. Baseline Characteristics

A descriptive analysis of baseline variables collected in section 9 of the CRF will be performed to compare the characteristics of participants in each treatment group (as described in Table 2, Table 3 and Table 4). Means and standard deviations, or medians and interquartile ranges will be reported for continuous variables. Frequencies and percentages will be reported for categorical variables.

Due to the inclusion of infants from multiple births, infants were nested within mothers/families. Some characteristics were measured at the infant level (e.g. infant sex), others were measured on the mother or family level (e.g. mother completed secondary education). For the purpose of summarising baseline and post-randomisation characteristics, variables measured on the infant will be presented on

this level (Table 2 and 4) and variables measured on the mother or family level will be presented on this level (Table 3).

Table 2. Infant baseline characteristics

Infant baseline characteristic	**CRF reference**	**Categories**
Sex	Section 1.1	Male Female
Gestational age	Section 1.1	<30 weeks 30 to 32 weeks
Tin colour randomised	Section 1.1	Purple Yellow Red Green
Postnatal age at randomisation, days	Section 1.1: randomisation date - infant date of birth	
Plurality	9.10	Singleton Twins Triplets
Female infants	Section 1.1	
Male infants	Section 1.1	
Birth order	9.11	Singleton First of a multiple birth Second of a multiple birth Third of a multiple birth
Infant gestational ages at birth	3.10	Number by completed week and mean, SD
Delivery mode	Section 9.9	
Vaginal	Section 9.9	
Instrumental	Section 9.9	
Vaginal birth - any	Vaginal+Instrumental	
Caesarean section in labour	Section 9.9	
Caesarean section no labor	Section 9.9	
Caesarean section (any)	Section 9.9 - Caesarean section in labour + Caesarean section no labor	

Infant baseline characteristic	CRF reference	Categories
Apgar score	9.12	1 minute 5 minutes
Birth weight	9.13	
Birth length	9.14	
Birth head circumference	9.15	
Surfactant	9.33	Unknown None Curosurf
Randomisation BUN	Blood tab	
Randomisation Creatinine	Blood tab	
Randomisation Albumin	Blood tab	
Randomisation Glucose	Blood tab	
Randomisation pH	Blood tab	
Randomisation base excess	Blood tab	
Postnatal age at which standard nursery fortifier commenced	1.6	
Postnatal age at which Poppet fortifier commenced	1.1	
Days of standard fortifier before starting Poppet fortifier	1.6-1.1	

Table 3. Maternal characteristics

Maternal baseline characteristic	CRF reference	Categories
Age, years	9.2	
Ethnicity of mother	9.3	
Smoked during pregnancy	3.6	Unknown No . Yes If yes, mean or mdian number per week

Maternal baseline characteristic	CRF reference	Categories
Alcohol during pregnancy	3.7	Unknown No Yes, Yes, mean or median number standard drinks per week
Highest level of secondary education	3.8	8 9 10 11 12 Unknown
Percentage completed secondary school	8+9+10+11 compared with 12	Y N
Highest level of further Study	3.9	No further study Certificate/Diploma (includes apprenticeships) Degree Higher degree Unknown
Further study (any)	3.9 (Certificate/Diploma (includes apprenticeships) + Degree + Higher degree	Y N
Assisted conception	9.4	Unknown None used for this pregnancy Hyperovulation – any hormone used to stimulate ovulation IVF/GIFT etc Other - infertility treatment not mentioned above, including artificial insemination
Assisted conception (any)	Hyperovulation – any hormone used to stimulate ovulation+ IVF/GIFT etc+ Other - infertility treatment not mentioned above, including artificial insemination	

Maternal baseline characteristic	CRF reference	Categories
Previous preterm births	9.5	Unknown No previous preterm birth Yes, there was a previous preterm birth
Previous perinatal death	9.6	Unknown No previous perinatal death Yes, has had a previous perinatal death
Presenting antenatal problem	9.7	Unknown, information not available Preterm pre-labour rupture of membranes Preterm labour Hypertension in pregnancy Antepartum haemorrhage Suspected intrauterine growth restriction Fetal distress Other None − No presenting problem. Baby must be born at term.
Antenatal corticosteriods	9.8	Unknown None – steroids not given < 24 hours - first dose given <24 hours prior to this baby's birth Complete – more than 1 dose of steroids given, and 1 dose at >24 hours and <8 days before birth Given at > 7 days before baby's birth

7.4. Serious Adverse Events

Descriptive information relating to serious adverse events will be presented by treatment group.

The following events are considered to be serious adverse events:

- Incidence of Necrotising Enterocolitis (NEC)
- Death

7.5. Missing Data

Missing data will be assessed descriptively by treatment group for each outcome variable specified in Section 7.

8. STATISTICAL ANALYSES

In this section the following details are provided for each outcome variable specified in Outcome Variables:

- Hypothesis – the hypothesis to be tested (for primary and major secondary outcomes)
- Outcome - a detailed description of the outcome variable, including the type of variable, the relevant variable(s) in the CRF and how it will be calculated (if applicable).
- Effect - the measure of treatment effect to be reported
- Analysis - the type of statistical analysis to be performed
- Adjustment - the baseline covariates (stratification variables and potential confounders) to adjust in the adjusted analyses
- Study end – removal of the feeding tube

For each outcome variable, statistical significance will be assessed at the 0.05 level using a two-sided comparative test of treatment effect, unless otherwise specified.

For binary outcomes, if the number of subjects experiencing the outcome is considered too small for the planned analysis to be sensible, a Fisher's exact test will be performed instead with no adjustment made for baseline covariates.

For analyses performed using a log binomial model, a log Poisson model with robust variance estimation will be used if the model fails to converge.\

8.1. Primary Outcome

8.1.1. Rate of weight gain, Anthropometrics G

Hypothesis	A high protein entree of human milk fortifier administered before each feed will improve the rate of weight gain.
Outcome	Continuous outcome measured daily from study start (randomisation) to study end (removal of feeding tube).
Effect	Difference in mean weight gain over study duration (high protein versus standard protein).
Analysis	Two stage model: Stage 1: A linear effects model with a random intercept and slope for each infant will be calculated to estimate each infant's mean gain in weight per day across the study period (then multiplied by 7 to estimate weekly weight gain). Stage 2- Fitting a linear regression model, using the slopes for each child from Stage 1 as the outcome in a linear regression model. Clustering within mother (i.e. multiple births) is accounted for by using a generalised estimating equation (GEE) with independence working correlation matrix. The stage 2

	model would be of the form:
	Weight gain per week (estimated at stage 1) =group + GA + sex
Adjustment	Gestational age, sex.

8.2. Secondary outcomes relating to the intervention

8.2.1. Rate of length gain, Anthropometrics H, I

Hypothesis	A high protein entree of human milk fortifier administered before each feed will improve the rate of length gain.
Outcome	Continuous outcome measured weekly from study start (randomisation) to study end (removal of feeding tube).
Effect	Difference in mean length gain over study duration (high protein minus standard protein).
Analysis	Two stage model:
	Stage 1: A linear effects model with a random intercept and slope for each infant will be calculated to estimate each infant's mean gain in length per week across the study period.
	Stage 2- Fitting a linear regression model, using the slopes for each child from Stage 1 as the outcome in a linear regression model. Clustering within mother (i.e. multiple births) is accounted for by using a generalised estimating equation (GEE) with independence working correlation matrix. The stage 2 model would be of the form:
	Length gain per week (estimated at stage 1) =group + GA + sex
Adjustment	Gestational age, sex.

8.2.2. Head circumference gain, Anthropometrics J, K

Hypothesis	A high protein entree of human milk fortifier administered before each feed will improve the rate of head circumference gain.
Outcome	Continuous outcome measured weekly from study start (randomisation) to study end (removal of feeding tube).
Effect	Difference in mean head circumference gain over study duration
Analysis	Two stage model:
	Stage 1: A linear effects model with a random intercept and slope for each infant will be calculated to estimate each infant's mean gain in head circumference per week across the study period.
	Stage 2- Fitting a linear regression model, using the slopes for

	each child from Stage 1 as the outcome in a linear regression model. Clustering within mother (i.e. multiple births) is accounted for by using a generalised estimating equation (GEE) with independence working correlation matrix. The stage 2 model would be of the form: Head Circumference gain per week (estimated at stage 1) =group + GA + sex
Adjustment	Gestational age, sex.

8.2.3. Lean (Fat Free) mass gain, ImpediMed output file

Hypothesis	A high protein entree of human milk fortifier administered before each feed will improve the rate of lean mass gain.
Outcome	Continuous outcome measured weekly from study start (randomisation) to study end (removal of feeding tube).
Effect	Difference in mean lean (fat free) mass gain over study duration
Analysis	Two stage model: Stage 1: A linear effects model with a random intercept and slope for each infant will be calculated to estimate each infant's mean fat free mass gain per week across the study period. Stage 2- Fitting a linear regression model, using the slopes for each child from Stage 1 as the outcome in a linear regression model. Clustering within mother (i.e. multiple births) is accounted for by using a generalised estimating equation (GEE) with independence working correlation matrix. The stage 2 model would be of the form: Fat Free Mass gain per week (estimated at stage 1) =group + GA + sex
Adjustment	GA, sex.

*8.2.3a ** Additional analyses**Change in Fat Mass, ImpediMed output file (NB this analysis is additional to those specified in the original analysis plan)*

Hypothesis	Infants in the high protein group will have a higher rate of fat mass decrease
Outcome	Continuous outcome measured weekly from study start (randomisation) to study end (removal of feeding tube)
Effect	Difference in mean fat mass over study duration
Analysis	Linear mixed effects model treating time (measure) as categorical, incorporating a group-by-measure interaction term, and an autoregressive correlation structure to account for correlation due to repeated measures over time. Mother id is included as a random effect to account for clustering.

*8.2.3b ** Additional analyses** Change in Fat Free Mass Proportion, ImpediMed output file (NB this analysis is additional to those specified in the original analysis plan).*

Hypothesis	Infants in the high protein group will have a higher percentage of lean mass over the duration of the study
Outcome	Continuous outcome measured weekly from study start (randomisation) to study end (removal of feeding tube)
Effect	Difference in fat free mass percent over study duration
Analysis	Linear mixed effects model treating time (measure) as categorical, incorporating a group-by-measure interaction term, and an autoregressive correlation structure to account for correlation due to repeated measures over time. Mother id is included as a random effect to account for clustering.

8.2.4. Small for gestational age (SGA) for weight on study end (tube out)

Hypothesis	Infants in the high protein group (experimental) will have a reduced incidence of SGA
Outcome	Binary outcome at study end - as measured against Beeby et al (1996) reference values
Effect	Relative risk of small for gestational age (high protein relative to standard protein).
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.2.5. SGA for length at study end

Hypothesis	Infants in the high protein group (experimental) will have a reduced incidence of SGA
Outcome	Binary outcome at study end
Effect	Relative risk of small for gestational age (high protein relative to standard protein) as measured against Beeby reference values.
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.2.6. SGA for head circumference at study end

Hypothesis	Infants in the high protein group (experimental) will have a reduced incidence of SGA

Outcome	Binary outcome at study end as measured against Beeby reference values.
Effect	Relative risk of small for gestational age (high protein relative to standard protein).
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.2.7. Study end weight, Anthropometrics G

Outcome	Weight at study end
Effect	Continuous
Analysis	Linear regression model, using a GEE to account for clustering at the mother level.
Adjustment	GA, sex.

8.2.8. Study end length, Anthropometrics H, I

Outcome	Length at study end
Effect	Continuous
Analysis	Linear regression model, using a GEE to account for clustering at the mother level.
Adjustment	GA, sex.

8.2.9. Study end Head circumference, Anthropometrics J, K

Outcome	Head circumference at study end
Effect	Continuous
Analysis	Linear regression model, using a GEE to account for clustering at the mother level.
Adjustment	GA, sex.

8.3. Secondary outcomes related to safety

8.3.1. Incidence of Necrotising Enterocolitis (NEC), Baby AO

Outcome	Binary outcome
Effect	Relative risk of NEC
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.3.2. Blood Urea Nitrogen, Blood

Outcome	Continuous outcome with repeated measures
Effect	Difference in BUN over study duration
Analysis	Linear mixed effects model treating time as categorical, incorporating a group-by-time interaction term, and an autoregressive correlation structure to account for correlation due to repeated measures over time. Mother id is included as a random effect to account for clustering.
Adjustment	GA, sex.

8.3.3. Albumin, Blood

Outcome	Continuous outcome with repeated measures
Effect	Value at each study week
Analysis	Linear mixed effects model treating time as categorical, incorporating a group-by-time interaction term, and an autoregressive correlation structure to account for correlation due to repeated measures over time. Mother id is included as a random effect to account for clustering.
Adjustment	GA, sex.

8.3.4. Creatinine, Blood

Outcome	Continuous outcome with repeated measures
Effect	Difference in Creatinine over study duration
Analysis	Linear mixed effects model treating time as categorical, incorporating a group-by-time interaction term, and an autoregressive correlation structure to account for correlation due to repeated measures over time. Mother id is included as a random effect to account for clustering.
Adjustment	GA, sex.

8.3.5. Glucose, Blood

Outcome	Continuous outcome with repeated measures
Effect	Difference in Glucose over study duration
Analysis	Linear mixed effects model treating time as categorical, incorporating a group-by-time interaction term, and an autoregressive correlation structure to account for correlation due to repeated measures over time. Mother id is included as a random effect to account for clustering.
Adjustment	GA, sex.

8.3.6. Base excess, Blood

Outcome	Continuous outcome with repeated measures

Effect	Difference in base excess over study duration
Analysis	Linear mixed effects model treating time as categorical, incorporating a group-by-time interaction term, and an autoregressive correlation structure to account for correlation due to repeated measures over time. Mother id is included as a random effect to account for clustering.AdjustmentGA, sex

8.3.7. pH, Blood

Outcome	Continuous outcome with repeated measures.
Effect	Difference in pH over study duration
Analysis	Linear mixed effects model treating time as categorical, incorporating a group-by-time interaction term, and an autoregressive correlation structure to account for correlation due to repeated measures over time. Mother id is included as a random effect to account for clustering.
Adjustment	GA, sex

8.3.8. Feeds supplemented (Protifar etc), Anthropometrics AB

Outcome	Binary outcome
Effect	Relative risk of feeds supplemented
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.3.9. Number of days feeds interrupted/held, Anthropometrics AA

Outcome	Count outcome (number of days on which feeds were interrupted/withheld)
Effect	Ratio of number of days feeds interrupted/withheld
Analysis	Log Poisson model, using a GEE with independence working correlation matrix to account for clustering at the mother level. If the Poisson model fails to converge, a negative binomial model will be fitted.
Adjustment	GA, sex.

8.3.10. Time taken to reach full enteral feeds (150 ml/kg/day), Baby U

Outcome	Count outcome (number of days to FEF)
Effect	Ratio of number of days to FEF between groups
Analysis	Log Poisson model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4. Secondary clinical outcomes

8.4.1. Surgery, Baby AS

Outcome	Binary outcome
Effect	Relative risk of any surgery
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.2. Early Onset Sepsis, Baby BC

Outcome	Binary outcome
Effect	Relative risk of early onset sepsis
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.3. Late Onset Sepsis, Baby BG

Outcome	Binary outcome
Effect	Relative risk of late onset sepsis
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.4. Postnatal steroids, Baby AW

Outcome	Binary outcome
Effect	Relative risk
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.5. Need for Nitric Oxide, Baby AH

Outcome	Binary outcome
Effect	Relative risk of need for NO
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.6. Need for any oxygen therapy, Baby AD

Outcome	Continuous outcome
Effect	Difference in mean/median
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.7. Home oxygen, Baby AF

Outcome	Binary outcome
Effect	Relative risk
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.8. Hours of IPPV, Baby AI

Outcome	Count outcome (number of hours of IPPV)
Effect	Ratio of number of hours of CPAP between groups
Analysis	Log Poisson model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.9. Hours of CPAP, Baby AJ

Outcome	Count outcome (number of hours of CPAP)
Effect	Ratio of number of hours of CPAP between groups
Analysis	Log Poisson model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.10. Hours of Optiflow, Baby AK

Outcome	Count outcome (number of hours of optiflow)
Effect	Ratio of number of hours of Optiflow between groups
Analysis	Log Poisson model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.1. Hours of Optiflow+CPAP+IPPV, Baby AI+AJ+AK

Outcome	Count outcome (number of hours of any oxygen)
Effect	Ratio of number of hours of any oxygen between groups

Analysis	Log Poisson model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.2. Need for HFOV (yes/no) , **Baby AG**

Outcome	Binary outcome
Effect	Relative risk of need for HFOV
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.3. Main respiratory diagnosis, **BabyAC**

Outcome	Binary outcome
Effect	Relative Risk of any main respiratory diagnosis
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.4. Chronic lung disease (oxygen at 36 weeks) , **Baby BK**

Outcome	Binary outcome
Effect	Relative risk of oxygen at 36 weeks gestation
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.5. Maximum grade IVH, **Baby AL**

Outcome	Binary outcome
Effect	Relative risk of any grade IVH
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.6. Cerebral cystic formations, **Baby AM**

Outcome	Binary outcome
Effect	Relative risk for any cystic formation
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.7. Retinopathy of prematurity, **Baby AN**

Outcome	Binary outcome
Effect	Relative risk for ROP
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.8. Days of parenteral feeds, Baby BM

Outcome	Count outcome (number of days)
Effect	Ratio of days of Parenteral feeds between groups
Analysis	Log Poisson model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.9. Days of intravenous lipid therapy, Baby BN

Outcome	Count outcome (number of days)
Effect	Ratio of days of IV lipids between groups
Analysis	Log Poisson model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

8.4.10. Babies on NED, Additional

Outcome	Binary outcome
Effect	Relative Risk of NED
Analysis	Log binomial model, using a GEE with independence working correlation matrix to account for clustering at the mother level.
Adjustment	GA, sex.

Appendix K - Per protocol analyses

A per protocol analysis was specified in the Protocol (Appendix) and Data Analysis Plan (Appendix) and included infants who had received at least 70% of their prescribed Poppet. Infants did not receive the Poppet supplement when a feed was greater than 50% formula, when feeds were held and if the infant was withdrawn from the study. Forty four (44) of the 60 randomised infants were assessed as Per Protocol. Per Protocol analyses for weight gain, length gain and head circumference gain are presented with Intention To Treat analyses in Section 4.6.3. This study was powered to detect a difference in weight gain with 60 randomised infants. Therefore findings in these data are to be interpreted with caution.

A1.1 Growth outcomes – Per Protocol

A1.1.1 Incidence of SGA at study end – Per Protocol

The Per Protocol analysis for incidence of SGA at study end reflected the results of the full data set (Table 4.11) such that there were no significant differences for incidence of SGA between the High protein and Standard infants at study end (Table A1.1). There were more infants classified SGA for weight and head circumference

Table A1.1 SGA at trial end – Per Protocol

			Unadjusted		Adjusted[a]	
	High protein n=21 n (%)	Standard n=23 n (%)	RR (95%CI)	p value	RR (95%CI)	p value
SGA for weight	7 (33.3)	3 (13.0)	2.6 (0.8, 7.8)	0.10	2.4 (0.7, 7.6)	0.15
SGA for length	14 (66.7)	11 (43.4)	1.5 (0.9, 2.7)	0.13	1.4 (0.8, 2.4)	0.27
SGA for head circ.	4 (19.1)	1 (4.4)	4.4 (0.8, 2.5)	0.09	3.7 (0.7, 2.0)	0.12

[a] Adjusted for sex and gestational age; p represents p value

A1.1.2 Fat free mass as a proportion of body weight – Per Protocol

Over the first four weeks of the trial, when >75% of participants were still in hospital, fat free mass as a proportion of body weight was increased in high protein infants from weeks one to four (p=0.04) but with no significant group by time interaction effect (p=0.83) (Figure A1.1).

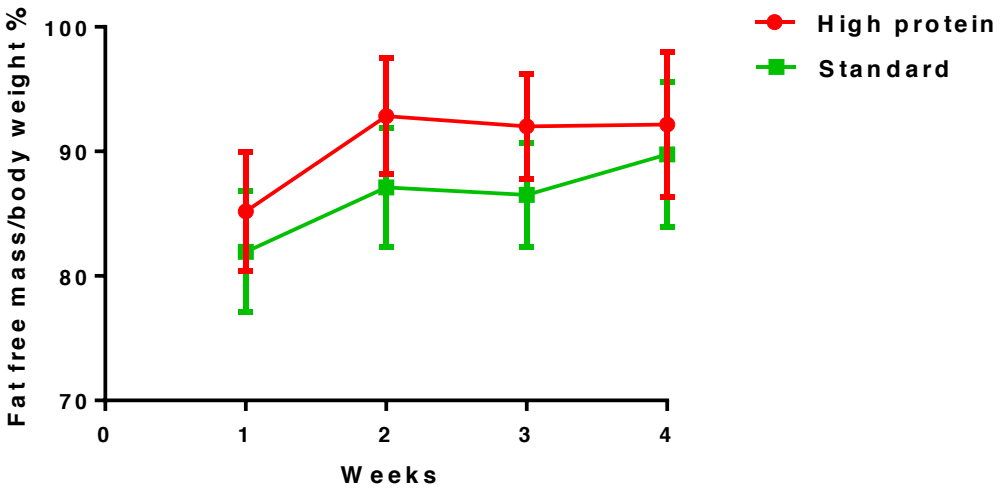


Figure A1.1 Fat free mass as a proportion of body weight for the first 4 weeks of the study. Adjusted p vales Group p=0.04, Time p<0.01, Group*time=0.83 Week 1 High protein n=21 Standard n=23, Week 2 High protein n=21 Standard n=23, Week 3 High protein n=21, Standard n=23, Week 4 High protein 19, Standard n=21.

A1.1.3 Weight, length and head circumference at study end – Per Protocol

There was no difference in the adjusted data for study end weight, length or head circumference. There was an observed difference in the unadjusted length at study end, such that Standard infants were longer.

Table A1.2 Anthropometric growth measures at study end – Per Protocol

			Unadjusted		Adjusted[a]	
	High protein n=21	Standard n=23	Difference	p value	Difference	p value
Weight (g)	2646 (2488,2805)	2814 (2674,2954)	-169 (-362, 25)	0.09	-157 (-341, 28)	0.10
Length (cm)	45.2 (44.5, 45.9)	46.3 (45.0, 47.0)	-1.1 (-2.1, -0.1)	0.04	-0.9 (-1.8, 0.1)	0.09
Head circ (cm)	33.3 (32.7, 33.9)	33.6 (33.0, 34.1)	-0.3 (-0.6, 0.7)	0.44	-0.2 (-0.6, 0.6)	0.66

Mean; p represents p value; CI 95%; [a] Adjusted for sex and gestational age.

A2.1 Clinical outcomes – Per Protocol

A2.1.1 Respiratory

Need for respiratory support was balanced between the groups (Table A2.1) with no significant outcomes.

Table A2.1 Need for respiratory support and incidence of chronic lung disease – Per Protocol

	High protein n (%), n=21	Standard n (%), n=23	Unadjusted RR mean (95% CI)	p value
Home oxygen	1 (4.8)	1 (4.4)	1.10 (0.08-15.12)	0.95
Need for HFOV	2 (6.5)	1 (6.9)	2.19 (0.16-30.23)	0.56
Nitric Oxide	0	1 (4.4)		0.99^
Chronic lung disease	1 (4.8)	1 (4.4)	1.10 (0.08-15.12)	0.95
Postnatal steroids	1 (4.8)	2 (8.7)	0.55 (0.06-5.90)	0.60

^Fisher's Exact Test

A2.1.2 Mean hours of ventilator mediated support – Per Protocol

Hours of respiratory therapy was not significantly different between the groups but trended to be higher in the High protein infants, reflecting the Intention to Treat analysis.

Table A2.2 Hours of IPPV, CPAP and HHFNC– Per Protocol

			Unadjusted		Adjusted[a]	
	High protein	Standard	Ratio	p	Ratio	
Hours of IPPV	11.5 (3.9, 6.1)	7.7 (2.9, 20.3)	1.5 (0.4, 6.1)	0.57	1.2 (0.4, 4.1)	0.77
Hours of CPAP	28.0 (17.8, 43.8)	28.6 (16.4, 49.5)	1.0 (0.5, 2.8)	0.96	1.0 (0.6, 2.6)	0.90
Hours of HHFNC	68.1 (22.0,211.0)	84 (30.5, 232.1)	0.81 (0.2, 3.6)	0.78	0.7 (0.2, 2.4)	0.52
Hours of Resp. Support	107.5 (49.3,234.6)	120.4 (54.6,265.3)	0.89 (0.5, 3.0)	0.83	0.8 (0.3, 1.9)	0.54

Mean; p represents p value; 95% CI; Number of infants who received IPPV, High protein n=2, Standard n=1, CPAP, High protein n=15, Standard n=14, HHFNC, High protein n=6, Standard n=9.[a] Adjusted for sex and gestational age

A2.1.3 Other clinical outcomes – Per Protocol

There were no significant differences in clinical outcomes.

Table A2.3 Other clinical outcomes – Per Protocol

			Unadjusted	
	High protein n=21, n (%)	Standard n=23, n (%)	RR (95% CI)	p
Any IVH[+]	3 (15)	0		0.23^
Any Cerebral Cyst[-]	0	1 (5)	1.22 (1.00, 1.48)	0.052
Surgery	2 (9.5)	1 (4.4)	1.40 (0.25, 7.85)	0.70[#]
NED participation*	8 (38.1)	7 (24.1)	1.20 (0.63, 2.30)	0.58[#]
Retinopathy of prematurity	0	1 (4.4)		0.43^
Late onset sepsis	1 (4.8)	0		0.48^

^ Fisher's Exact; [#] Unadjusted analyses only due to small n numbers; [+] High protein n=20, Standard n=19, [-] High protein n=20, Standard n=18, p represents p value; [a] Adjusted for sex and gestational age

*NED (Neonatal Early Discharge) allows infants to go home with a naso-gastric feeding tube and is offered to families on a case by case basis. Eligibility criteria include but are not limited to, good weight gain, ability of mother to administer gavage feeds and that the family live in close proximity to WCH. The infant is visited by a neonatal nurse each as frequently as required, typically daily in the first instance. Mothers in the NED program were taught to administer the trial product and kept a record of compliance and fluid intake.