The Efficiency and Safety of a Higher Protein Human Milk Fortifier on Growth for Preterm Human Milk-fed Infants.

Jacqueline Miller

This thesis is submitted for the degree of Doctor of Philosophy

School of Paediatrics and Reproductive Health

Faculty of Health Science

The University of Adelaide

May 2010

Table of Contents

Abbreviations

AGA	Appropriate for gestational age
ANZNN	Australian & New Zealand Neonatal Network
AOAC	Association of Official Analytical Chemists
BCAA	Branch chain amino acids
BUN	Blood urea nitrogen
BW	Birth weight
CHO	Carbohydrate
CI	Confidence interval
CLD	Chronic lung disease
CNRC	Child Nutrition Research Centre
CONSORT	Consolidated Standards of Reporting Trials
CPAP	Continuous positive airway pressure
CSUN	Corrected serum urea nitrogen
CV	Coefficient of variation
D/C	Discharge
DMAC	Data Management and Analysis Centre
EBM	Expressed breast milk
EDD	Estimated date of delivery
ELBW	Extremely low birth weight
FMC	Flinders Medical Centre
GA	Gestational age
GEE	Generalised estimating equations
GIT	Gastro-intestinal tract
HMF	Human milk fortifier
IQ	Intelligence quotient
IR	Infra-red
IUGR	Intra-uterine growth retardation
IV	Inverse variance
IVF	In-vitro fertilisation
IVH	Intraventricular haemorrhage

LBW	Low birth weight
MDI	Motor developmental indices
NATA	National Association of Testing Authorities
NEC	Necrotising enterocolitis
NED	Neonatal Early Discharge
NICU	Neonatal Intensive Care Unit
NMI	National Measurement Institute
NNT	Number needed to treat
NPN	Non-protein nitrogen
OHC	Occipital head circumference
PDA	Patent ductus arteriosis
PDI	Psychomotor developmental indices
PMA	Post menstrual age
PVL	Periventricular leukomalacia
QL	Quantitation limit
RCT	Randomised controlled trial
ROP	Retinopathy of prematurity
SCBU	Special Care Baby Unit
SD	Standard deviation *or* Study day
SE	Standard error
SES	Socio-economic status
SGA	Small for gestational age
SUN	Serum urea nitrogen
TEM	Technical error of measurement
TGA	Therapeutic Goods Administration
VLBW	Very low birth weight
WCH	Women's and Children's Hospital
WHO	World Health Organisation
WMD	Weighted mean difference

List of Tables

List of Figures

Abstract

Preterm births represent approximately 8% of births in Australia, and this rate has been increasing over the last decade. Nutrition is a cornerstone of their medical management, yet very premature infants remain difficult to adequately nourish and growth failure is a common consequence of prematurity. Human milk is the preferred feed but has inadequate protein to meet their high requirements and must be fortified. Commercial fortifiers contain conservative amounts of protein and fail to compensate for the fall in the protein content of expressed breast milk over time. This thesis tested the hypothesis that preterm infants fed human milk with a higher protein fortifier (1.4 g/100 mL) would have greater length gain with no metabolic disturbances when compared to infants fed human milk fortified to standard levels (1.0 g protein/100 mL).

In a randomised controlled trial infants born <31 weeks gestation, whose mothers intended to provide breast milk for their infants, were randomly allocated to receive either the experimental fortifier containing 1.4 g protein or a fortifier equivalent to standard care, containing 1 g protein. The fortifiers were manufactured specifically for the study and were made isocaloric by adjusting the carbohydrate content. They were identical in appearance and mixing rates and all personnel involved in the trial were blinded to the allocation. Preterm formula was used if breast milk supply was inadequate. The intervention period was from the start of fortification to discharge or the infant's estimated due date, whichever came first. The primary outcome was length gain (cm/week) and secondary

outcomes included other growth measurements (weight and head circumference gain), biochemical markers (urea nitrogen, creatinine, albumin, pH, amino acids) and data describing their clinical course during the hospital admission.

There was a slight improvement in length gain in the higher protein group but this did not reach statistical significance (mean (95% CI) 1.15 (1.10-1.19) and 1.09 (1.05-1.13) cm/week in the higher and standard groups respectively, $p = 0.08$). However, fewer infants were classified as small for gestational age for length at discharge in the higher protein group (49% versus 63% in the higher and standard protein groups, respectively, $p = 0.04$). There were no differences in weight or head circumference gain between the groups. Serum urea nitrogen concentrations and whole blood amino acid levels were higher in the higher protein group but plasma albumin, creatinine and pH were not different between groups. There were no differences in clinical outcomes such as retinopathy of prematurity, sepsis, necrotising enterocolitis, number of infants requiring surgery or length of hospital stay.

A higher protein human milk fortifier appears to be well tolerated and safe to use in preterm human milk fed infants born <31 weeks gestation. The extra protein protects against a classification of small for gestational age for length at discharge and may improve length gain. Further studies directed toward comparisons between fortifiers with levels of protein >1 g/100 mL are required to determine the optimum protein concentration of fortifiers.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma 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. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines.

Jacqueline Miller

Acknowledgements

The idea for this thesis was conceived jointly by members of the Women's and Children's Hospital (WCH) Neonatal Research Group: Prof Maria Makrides, Prof Robert Gibson, Dr Carmel Collins and Dr Andrew McPhee. I thank them all for the opportunity to undertake my thesis in this area and for using their influence to make this project a reality.

The Neonatal Units at both WCH and Flinders Medical Centre (FMC) played an active role in this project. I am very grateful to all staff for their input; for welcoming me in to their specialised environment and allowing me to undertake this research. In particular, I would like to acknowledge Dr Andrew McPhee, Prof Ross Haslam, Dr Scott Morris, the research midwives Ros Lontis, Louise Goodchild, Cheryl Chambers and Milk Room staff.

Statistical analyses were undertaken by the Data Management and Analysis Centre (DMAC) at The University of Adelaide and I am very grateful to members of this department and in particular Tyman Stanford.

Amino acid blood analyses were performed by the WCH Neonatal Screening Laboratory and I would like to thank Enzo Ranieri and his team for their work in this area.

Financial support was provided in the form of an MS McLeod scholarship from the WCH Foundation and the Denis Harwood Grant-in-Aid. Trial products were generously donated by Nestlé.

On a personal note, there are a large number of people who have supported me during my candidature; far too many to list. To all of these friends, colleagues and family, thank you very much for your support and encouragement over this time.
I would also like to thank my supervisors, Prof Maria Makrides, Dr Carmel Collins and Prof Robert Gibson, for their expertise, guidance and considerable support.

Finally, I am enormously grateful to the families who participated in this study. Without them, none of this would have been possible.

Chapter 1 Literature review

1.1 Preterm birth

Preterm birth, defined as birth before 37 weeks of gestation, is a major public health issue for both developing and industrialised societies. In wealthy countries, the incidence is approximately 10% of all births and has been increasing since the mid 1990s (1-3). Australian preterm births accounted for 8.1% of births in 2007, with:

- 6.4% of births between 32 and 36 weeks gestation
- 0.8% between 28 and 31 weeks gestation and
- 0.9% between 20 and 27 weeks gestation (4).

Recent advances in neonatal medicine have led to higher survival rates of preterm infants, particularly the smaller and more immature infants, defined as very low birth weight (VLBW), weighing <1500 g and extremely low birth weight (ELBW) weighing <1000 g. While these infants represent only a small fraction of preterm births, they account for a high proportion of the health care budget. Due to their immaturity, they frequently have a complex clinical course which can impact on their subsequent growth and cognitive development. These problems extend far beyond the neonatal period and can result in significant childhood disability such as long term motor, cognitive, visual and hearing problems, as well as more subtle problems such as behavioural and learning disorders (5). The cost to communities is large, both emotionally and economically, and affects many levels of society such as families, education, and health and welfare systems. In 2005 the USA economic burden associated with preterm birth was estimated to be US $26.2

billion (6). A UK cost estimate model estimated the expenditure related to preterm birth in England and Wales in 2006 to be £2.946 billion (US $4.567 billion) (7). This analysis also identified an inverse relationship between gestational age at birth and cost with very (<33 weeks) and extremely (<28 weeks) preterm birth children costing significantly more than term or the average preterm child.

Nutrition is one of the cornerstones of treatment for preterm infants. Better growth indicates better nutrition and is important to neonatologists because of the influence it has on both short and long term outcomes. Short term health outcomes such as growth, neurodevelopment, necrotising enterocolitis (NEC) and infection are known to be influenced by the type of feed (8), the rate of feeding (9, 10) and nutrient density of the milk (11, 12). Early studies in animal models demonstrated that restricting feeding during sensitive periods of development not only limits growth but also has the ability to programme the neonate to lifelong poor growth (13). Similarly, epidemiological evidence in infants suggests that poor growth, and therefore under-nutrition, in fetal and early postnatal life may increase central adiposity in adulthood (14, 15) and risk of cardiovascular disease (16, 17), particularly if there is rapid growth during early childhood (18, 19). Equally, later neurodevelopment is adversely influenced by under-nutrition in the neonatal period (20-22). Dietary intake must therefore be balanced to provide enough nutrients for normal organ development, especially the brain, while at the same time avoiding excesses that may cause metabolic imbalances. Improved nutritional management is therefore vital to achieve better outcomes for these infants but is also very challenging to attain. Due to an immature gastro-intestinal

tract, the infant cannot initially cope with the high level of nutrients he/she requires. This creates a period of time when requirements are not being met, thus parenteral nutrition is required and enteral feeds are graded up gradually to meet requirements. Similarly, preterm infant kidneys and immune function are not fully functional and clinicians must walk a tightrope between providing them with enough nutrition to adequately support growth without unduly stressing organ systems. There is a large amount of evidence to suggest that protein requirements are not met by current feeding practices and that protein, more so than energy, is the limiting nutrient regarding growth in extremely and very preterm infants (12, 23-27).

This review aims to evaluate the literature concerning the effect of nutrition, specifically protein, in human milk fed preterm infants on growth, in order to inform the development of a nutrition intervention designed to improve growth.

1.2 Nutritional management of preterm infants

A generally accepted goal for nutritional management of preterm infants is to provide sufficient nutrients to achieve growth rates comparable to those *in utero* (28). In addition to absolute weight gain, the relative contributions of lean body tissue and fat mass to weight gain are known to be different between preterm infants and the fetus at a comparable stage of development and the significance of this is unknown (29). *In utero* conditions are designed for rapid tissue accretion with an uninterrupted supply of predigested nutrients available via the placenta. This situation is quite difficult to mimic postnatally because, while nutrient requirements remain high, the rate of delivery of these nutrients is limited by the immaturity of the gastrointestinal tract (GIT) and other organs such as the liver

and kidney which are involved in the digestion, absorption and metabolism of nutrients. There is a lag period while the GIT matures and infants accrue a nutrient deficit which must later be replaced. Under-nutrition during this time translates into poor growth (25).

1.2.1 Growth and neuro-development of preterm infants

Growth of preterm infants is generally thought to be suboptimal and is often considered to be an inevitable consequence of prematurity (25, 30-34). While some weight loss at birth is a normal physiological process, losses in ELBW infants are associated with protein catabolism rather than just fluid shifts and are known to be larger than in more mature infants (35, 36). These infants also take longer to regain birth weight and fail to adequately catch up this deficit during the hospital admission (37-39).

Ehrenkranz *et al* developed growth curves characterising contemporary growth from a large group of appropriate for gestational age (AGA) VLBW infants (n = 1660) in the mid 1990s (31). **Figure 1-1** shows a comparison of these with intra-uterine growth curves and demonstrates that, despite an appropriate mean birth weight, these infants initially lost weight and that even when stable growth was established, grew at a slower rate than *in utero*. The result of this was that the majority of the infants became growth restricted and were below the 10th percentile for weight by 36 weeks post menstrual age (PMA). Infants born less mature grew at a slower rate than more mature infants and there was a substantial shift of the growth curve to the right in the least mature infants born at 24 to 25 weeks gestation.

Figure 1-1 Preterm growth curves compared with intra-uterine growth Adapted from Ehrenkranz (31)

This finding of generalised poor growth in preterm infants has been confirmed by others (25, 30, 38, 40-42) leading to it being described as a universal or inevitable problem.

We undertook an audit of growth and dietary intake at the Women's and Children's Hospital (WCH) neonatal unit to determine the degree of growth restriction in our population (33). The infants audited were <33 weeks gestation and were born between 2001 and 2003, approximately 6-8 years later than the population studied by Ehrenkranz *et al* (31).

Table 1-1 Small for gestational age (SGA) rates at birth & discharge at WCH

	At birth		At discharge	
	<33 weeks GA[a] (n =138)	**<28 weeks GA**[b] (n = 35)	**<33 weeks GA** [a] (n =137)	**<28 weeks GA**[b] (n = 35)
Weight <10th percentile, n (%)	11, (8)	5, (14)	51 (37)	15 (43)
Length<10th percentile, n (%)	18 (13)	1 (3)	76 (55)	27 (77)

[a]Whole cohort of infants (<33 weeks GA) [b]Subset of infants born <28 weeks GA

Table 1-1 shows that approximately one third of infants had a discharge weight of <10th percentile and 55% had a discharge length of <10th percentile (33). Thus, despite the advances made in nutritional management since the Ehrenkranz audit (31), we were still unable to adequately support growth in at least one third of these infants.

This suboptimal growth of preterm infants is a marker for malnutrition and is a major concern because it occurs during a critical period of brain development equivalent to the last trimester of pregnancy. There is evidence to suggest that very preterm infants have cognitive and behaviour problems extending into school age and early adulthood with reported school difficulties (43-45), psychiatric issues during adolescence (46) and educational disadvantages in early adulthood (47). There is also evidence that birth weight plays a role in these effects with infants with a birth weight <1000 g showing poorer performance than heavier preterm infants (43). Other studies have shown a positive relationship between weight gain, head growth and neurodevelopmental outcomes (45, 48-51). What proportion of these effects can be related to early nutrition is difficult to establish

as brain development and behaviour are complex outcomes affected by a multitude of factors. Also, these follow-up studies relate to people who were premature many years ago when nutritional management may have been different to current practice. However, two dietary factors known to affect neuro-development are the nutrient content of the feed and the use of human milk. Lucas *et al* demonstrated a positive effect on neurodevelopment of preterm infants fed nutrient enriched formula versus a term formula or unfortified expressed breast milk (EBM) in the neonatal period (21). These results were more pronounced in male infants and those born small for gestational age (SGA). In a later study by the same author, donor milk was compared with preterm formula, either as the sole feed or as a supplement to maternal EBM (52). No differences in Bayley's psychomotor and mental development indices (PDI & MDI) were found between the groups despite the lower nutrient content of the donor milk, suggesting that human milk may compensate in some way for the lack of nutrients. Lucas *et al* then compared the donor milk group with the standard formula group from the first study and found human milk fed infants had higher PDI and MDI scores despite the lower nutrient content of the donor milk compared with standard term formula (53). These findings suggest that human milk is preferable for the neurodevelopment of preterm infants. This is supported by a meta-analysis investigating the effect of human milk versus formula feeding on cognitive development at 6 – 23 months of age (54). This showed an advantage of human milk feeding of 2.6 IQ points for normal weight infants, whereas the advantage increased to 5.1 IQ points for low birth weight infants.

It seems likely therefore that both under-nutrition and type of feeding during a critical period of brain growth equivalent to the last trimester of pregnancy will have some effect on neurodevelopment and this effect has the capacity to last into adult life. As growth is a marker of nutritional intake, ensuring that infants grow appropriately and meet their nutritional requirements during this period would thus seem a good strategy for optimising later health outcomes and perhaps preventing later neuro-developmental problems.

1.2.2 Protein and energy requirements

Nutrient requirements for very preterm infants are not well defined. This heterogeneous group of infants varies significantly in their length of gestation, birth weight and appropriateness of weight for gestational age, all factors which impact on nutrient requirements. They have varying (but generally low) reserves of energy (both fat and carbohydrate), a higher metabolic rate and higher protein turnover than term infants (27). They experience an increased rate of clinical events such as respiratory distress and infection which are known to alter nutrient requirements. It is probable that an individual infant's requirements will change during the course of the admission as he/she grows. It is therefore unlikely that a single set of recommendations will cover all preterm infants. Most professional organisations which have defined nutrient requirements have used either a range (55) or subdivide preterm infants into birth weight categories (28, 56). These requirements were set using the factorial method and fetal accretion rates. More recently, requirements have been re-evaluated, taking into account recent experimental evidence as well as factorial estimates, in a report commissioned by the American Food and Drug Administration in 2002 and undertaken by the Life Sciences Research Office of the American Society for Nutritional Sciences (12).

Similarly, a recent comprehensive summary of the scientific evidence was done by Tsang *et al* (57) who edited 'Nutrition of the Preterm Infant: Scientific Basis and Practical Guidelines'. Both of these reviews suggest an increase in protein intake is needed to meet requirements and the experimental evidence is summarised below in **Sections 1.2.2.1, 1.2.2.2 and 1.2.2.5**.

Protein requirements must be considered in relation to energy requirements and this is particularly so for very preterm infants who have very little carbohydrate stores and are at risk of amino acids being used for gluconeogenesis in the absence of sufficient energy. Protein energy ratios are therefore important to define and may also impact on the proportions of fat and lean tissue accretion.

Table 1-2 Summary of recommended nutrient requirements for preterm infants

Source	**Recommended Protein Intake g/kg/day**		**Recommended Energy Intake Kcal/kg/day**	
American Academy of Pediatrics 1985(28)	**Weight 800 – 1200 g** 4 3.1 g/100 Kcal	**Weight 1200 – 1800 g** 3.5 2.7 g/100 Kcal	120 (Range 105 – 130)	
ESPGAN[a] 1987 (55)	2.9 – 4 2.25 – 3.1 g/100 Kcal		130 (Range 110 – 165) Energy density of 65 – 85 Kcal/100 mL	
Canadian Pediatric Society 1991 (56)	**Transition 0-7 days** 1.0 – 3.0	**Stable to D/C** 3.5 – 4 (<1000 g) 3.0-3.6 (>1000 g)	**Transition 0-7 days** 70 - 80	**Stable to D/C** 105 – 135
Klein, 'Nutrient requirements of preterm infant formulas' 2002(12)	3.4 – 4.3 2.5 – 3.6 g/100 Kcal		110-135 Energy density of 67 – 94 Kcal/100 ml	
Tsang, 'Nutrition of the Preterm Infant' 2005(57)	'Reasonable nutrient intakes' for growing infants / enteral nutrition			
	ELBW 3.8 – 4.4 2.5 – 3.4 g/100 Kcal	**VLBW** 3.4 – 4.2 2.6 – 3.8 g/100 Kcal	**ELBW** 130 – 150	**VLBW** 110 – 130

[a] ESPGAN = European Society for Paediatric Gastroenterology and Nutrition; D/C = discharge

Table 1-2 summarises nutrient recommendations from key bodies and texts. In general the smaller infants have the higher requirement for protein and, taking into account recent evidence, there has been a tendency for requirements to be revised upwards over the last two decades.

The next three sections of this chapter review the literature concerning the protein content of various milk feeding regimes for preterm infants on growth. **Section 1.2.2.1** discusses trials which compared human milk with protein supplemented human milk, **Section 1.2.2.2** discusses trials comparing human milk with formula and **Section 1.2.2.5** discusses trials comparing formula of different protein concentrations.

1.2.2.1 Protein supplementation of human milk studies

Kuschel and Harding have published a series of Cochrane reviews on macronutrient supplementation of human milk for preterm infants (58-61). The review dealing specifically with protein supplementation (58) included 4 studies which compared protein supplementation of human milk with unfortified human milk (62-65). Ronnholm *et al* (62) and Boehm *et al* (65) used protein isolated from human milk as the supplement. Polberger *et al* (64) had 4 arms to their study as they compared unfortified milk, with milk fortified with either human milk protein, fat or a combination of both fat and protein. Only the unsupplemented (n = 8) and protein supplemented (n = 9) groups were included in the review. Putet *et al* (63) used a casein hydrolysate supplement. The findings of this review were that protein supplementation resulted in small but significant improvements in weight gain, (weighted mean difference (WMD) 3.6 g/kg/day, 95% CI 2.4 to 4.8 g/kg/day, p <0.00001) linear growth (WMD 0.28 cm/week, 95% CI 0.18 to 0.38

cm/week, p <0.00001) and head growth (WMD 0.15 cm/week, 95% CI 0.06 to 0.23 cm/week, p = 0.0006). Blood urea levels were higher in the fortified group but considered within the normal range (WMD 1.0 mmol/L, 95% CI 0.8 to 1.2 mmol/L, p <0.00001). While this review provides strong evidence in support of protein supplementation increasing growth, the comparisons were not isocaloric and may have also differed in micro-nutrients that are aligned with protein. Premature infants may be depleted of some of these nutrients and therefore a change in intake could also affect growth.

Further evidence in favour of protein supplementation is provided by Kuschel's and Harding's review on multi-component fortification (61). This review included 13 studies comparing unsupplemented human milk with milk fortified with protein plus either carbohydrate, fat or both (64, 66-76). The addition of vitamins and minerals was also common. Multicomponent fortification resulted in small increases in weight gain (WMD 2.3 g/kg/day, 95% CI, 1.7 to 2.9 g/kg/day), length gain, (WMD 0.12 cm/week, 95% CI, 0.07 to 0.18 cm/week) and head circumference gain (WMD 0.12 cm/week, 95% CI 0.07 to 0.16 cm/week).

Both of these reviews suggest that fortification with either protein, or multi-nutrient where protein is a major component, improve growth in preterm infants. However, these studies were not designed to be isocaloric, and varied several other nutrients in addition to protein so that it is not possible to tell if the effects seen are due to protein alone.

1.2.2.2 Human milk compared with formula studies

Several studies have compared human milk (either preterm milk or banked pooled term milk) with formula resulting in differing intakes of protein and, sometimes also, energy. Those studies that have non-isocalorically compared human milk with formula have undertaken assessments for growth (52, 77-80), nutrient balance (78-80) and neurodevelopment (52). In all of these studies both the protein and the energy intakes were higher in the formula fed group and, not surprisingly, they have all shown improved growth when fed formula. However, once again, protein was not the only variable. This remainder of this section will focus on trials that have compared milks with different protein concentrations but the same energy content.

1.2.2.3 Trials comparing unfortified human milk with formula

Two trials have compared unfortified human milk (with a low protein concentration) with infant formula of a higher protein content while maintaining the same energy intake (81, 82). Davies (82) compared EBM with a standard term formula. He found greater weight gain, although not significant, in the formula (higher protein) group compared with the EBM group. He also found significantly greater length gain in the subgroup of infants born 28 -32 weeks gestation in the group receiving the higher protein formula compared to EBM. However, this early study of 68 preterm infants was of poor quality. Although it was randomised, it is not clear whether the allocation was concealed, it was not blinded, attrition rate was not reported and the growth measurements were only taken at 3 time points over the 2 month study period. Svenningsen *et al* (81) also compared mother's own milk (1.6 g protein/100Kcal) with 2 formulae (2.3 and 3 g

protein /100Kcal) in a group of 48 VLBW infants. All 3 dietary groups had the same energy intake. Svenningsen *et al* found slightly improved weight gain with the higher protein formulae but no difference in length gain.

1.2.2.4 Trials comparing fortified human milk with formula

Two other studies have compared fortified human milk with formula at constant energy intakes (83, 84). Raschko *et al* (84) conducted a metabolic balance study and compared a group of 10 VLBW infants receiving EBM fortified with a liquid fortifier (1.8 g protein/100 mL) with another 10 infants receiving preterm infant formula (2.3 g protein/100 mL). The two feeding regimes were fed at rates to ensure the same energy intake. There was no difference in growth between the groups over the very short study period (8 days). Schanler *et al* (83) conducted the largest trial of fortified human milk (Enfamil HMF, Mead Johnson) compared with preterm formula (Enfamil Premature Formula 24, Mead Johnson) in a group of preterm infants in the USA in 1999. Both dietary regimes provided the same energy. This study was confounded by the fact that the fortified human milk group received some preterm formula as a supplement if EBM was unavailable. Outcomes were compared in those 108 infants fed >50 mL/kg/day of human milk (n = 62) with those fed exclusively preterm formula (n = 46). The study was not able to be blinded and it is not stated if the investigators were blinded to the outcome measures. Infants in the fortified human milk group consumed a higher volume of milk (180 vs 157 mL/kg/day) and also had a higher protein intake (4.1 vs 3.5 g/kg/day) but a similar energy intake (134 vs 129 Kcal/kg/day). Despite this, the human milk group achieved slower weight gain (22 vs 26 g/kg/day), slower length gain (0.8 vs 1.0 cm/wk) and smaller increases in the sum of skinfold

measurement (0.86 vs 1.23 mm/week). Schanler *et al* found lower fat absorption in the fortified human milk group, possibly due to interactions between lipid in human milk and the minerals in the fortifier and that fat absorption correlated significantly with weight gain. However, the human milk fed group showed other advantages such as a decreased length of stay (73 ± 19 vs 88 ± 47 days) and a lower combined incidence of NEC or late onset sepsis (31% vs 54%, $p \leq 0.01$) (83).

Studies comparing human milk with formula show varying effects of increasing protein on growth and this is likely to reflect the differences in study design. The potential nutrient interactions between fortifier and human milk and the intrinsic difference in the nutritional matrix of human milk versus formula highlight the complexity of interpreting these studies.

1.2.2.5 Studies comparing formulae of different protein levels

Studies comparing formulae give more insight into the effects of protein on growth because nutrients in the formula can be more tightly controlled than in human milk. It is also easier to identify, in advance, a cohort of exclusively formula fed infants and volume of intake can be more readily controlled. Several nutrient balance studies (11, 85-87) and growth studies (85, 88-90) have compared different protein intakes with energy levels held constant and have shown an increase in growth with higher protein intakes up to an intake of 4.6 g/kg/day. One early study on 304 low birth weight (LBW) infants (<2000 g) in 1969 by Goldman *et al* (88) randomised infants to two experimental diets containing either 2 or 4 g protein/100 mL (n = 152 in each group) and the same energy (80

Kcal/100 mL). This resulted in a large variation in protein intake (3 – 3.6 vs 6.0 – 7.2 g/kg/day). No differences in growth between the two groups were reported. The high protein group had higher plasma protein levels and decreased oedema but also showed some adverse effects of high protein intakes such as an increased rate of fever, lethargy and poor feeding. It is probable that this extreme level of protein intake exceeds requirements and also the upper safe level of intake which could interfere with growth, thereby failing to show any effect. Goldman *et al's* early study has several limitations. While their group of infants had birth weights <2000g, gestational age at birth was not reported and it is doubtful if these infants were comparable to today's very premature infants. The two feeding regimes were prepared by diluting a Similac liquid with water (56 to 100mL) and adding either lactose or casein to make the lower and higher protein feeds. This would have also diluted other nutrients which may have the potential to affect growth.

Kashyap *et al* performed a series of studies in which the protein to energy ratios of formulae were systematically varied with an aim to better define protein requirements. One of these studies (89) compared a total of 34 low birth weight (<2500 g) infants fed three different formulae at the following protein and energy intakes; Group 1, 2.24 g protein and 115 Kcal (n = 11) ; Group 2, 3.6 g protein and 115 Kcal (n = 11) and Group 3, 3.5 g protein and 149 Kcal (n = 12). Weight and head circumference gains were found to be statistically significantly lower in Group 1 (p value not reported) compared with the other two groups. Length gain showed the same trend but was not significant. The authors concluded that the lowest protein intake was inadequate as it did not support intra-uterine growth rates. They therefore repeated the experiment using higher protein intakes (85).

The three groups compared in this later trial had protein and energy intakes, respectively, of: Group 1, 2.8 g/kg/day and 119 Kcal/kg/day (n = 16); Group 2, 3.8 g/kg/day and 120 Kcal/kg/day (n = 16); and Group 3, 3.9 g/kg/day and 142 Kcal/kg/day (n = 18). When the two isocaloric groups were compared (groups 1 and 2) weight gain and nitrogen retention were significantly greater in the higher protein group (p value not reported). Length and head circumference showed a trend to higher gains with increasing protein intake but this was not statistically significant. Blood urea nitrogen (BUN) and amino acid levels paralleled protein intake. The increase in energy to 142 Kcal/kg/day in Group 3 resulted in higher fat accretion, estimated from nitrogen and energy balance data. In both the studies by Kashyap *et al*, infants were randomly allocated although the method and concealment of allocation were not reported. The ready to feed formulae were colour coded to facilitate blinding. The number of infants in groups in both studies is relatively small and although, taken overall, the data is suggestive, there is a need for higher quality trials with larger numbers for robust conclusions.

A recent study by Embleton *et al* (90) compared three isocaloric formulae with varying protein content (3.3 g/100 Kcal (n = 25), 3.0 g/100 Kcal (n = 26) and 2.7 g/100 Kcal (n = 26)) in preterm infants ≤34 weeks gestation and studied the effects on growth and body composition until term + 12 weeks corrected age. The study was randomised with adequate allocation concealment, and blinding was achieved by colour coding of the ready to feed formula. One infant died and another two were re-hospitalised after discharge and data up to these time points were included in the analyses. At discharge, infants with the highest protein intake were heavier, longer and had greater head circumference than the other two

groups ($p < 0.017$), however, the authors caution that these results were confounded by the fact that they were 6-7 postnatal days older than the other groups. Interestingly, the differences in growth were no longer apparent at term + 12 weeks corrected age, possibly indicating that the early weeks of life are more receptive to changes in protein concentration.

Nutrient balance studies have also been performed comparing protein intakes isocalorically and determining the effect on nitrogen accretion and weight gain. All of the three balance studies performed (11, 86, 87) showed increases in both weight gain and nitrogen accretion with a higher protein intake despite the variations in protein intakes used: 2.2 vs 3.6 g/kg/day (87); 2.7 vs 3.4 g/kg/day (86) and 3.8 vs 4.6 g/kg/day (11). All studies used a similar energy intake in the range of 110 to 120 Kcal/kg/day, which was unlikely to limit protein utilisation. The most recent study restricted eligible infants to ≤32 weeks gestation, as they were considered to be the most vulnerable group (11), whereas the other studies included gestations of 27 to 37 weeks (87) and 28 to 35 weeks (86). All studies had small numbers in each group ranging from 5 to 9 infants. Only one study was both adequately blinded and randomised (11), while Wauben *et al* (86) was randomised but did not report blinding and Schulze *et al* (87) was blinded but the method of allocation concealment was not reported. Despite these methodological differences, it would seem that increasing the protein intake of infants across a relatively wide range, may increase nitrogen accretion and increase weight gain.

The literature comparing formulae isocalorically provides the strongest evidence that increasing protein intake will improve growth and lean body mass gain. It is

interesting to note that the most recent study by Embleton *et al* (90) used a formula with 3.3 g protein/100 Kcal: a level higher than current preterm formula. While study results may have been confounded by older infants in the higher protein group, protein intakes were found to be safe with no evidence of acidosis, high ammonia or amino acid levels.

The next section of this review examines protein intakes and compares these with requirements.

1.2.3 Actual protein intakes

A number of studies have audited actual protein intakes in neonatal units (25, 30, 34, 91, 92) and these have been summarised in **Figure 1-2**. The delay in establishing full feeding leads to nutrient intakes which are frequently below the recommended requirements.

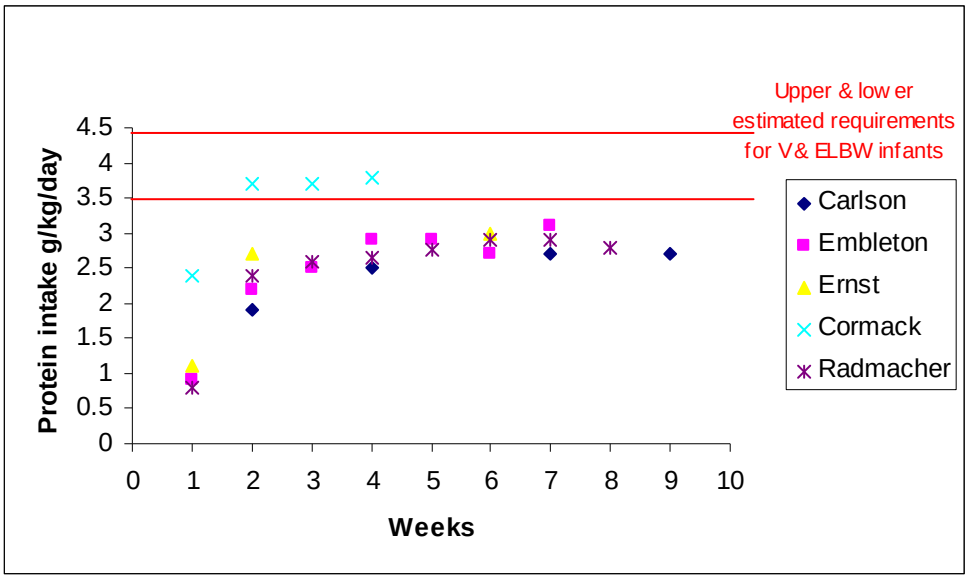


Figure 1-2. Summary of studies auditing protein intakes in neonatal units
Legend identifies primary author: see references (25, 30, 34, 91, 92)

Carlson's survey (91) in the late 1990s was limited to infants born weighing <1300 g and averaged the intake over the first two weeks, from 15 to 35 days, 36

to 56 days and 57 days to term. Protein intakes remained well below requirements for the duration of the study. Embleton *et al* (25) studied the cumulative nutrient deficits accrued in preterm infants ≤34 weeks gestation. Data shown in **Figure 1-2** are actual intakes for the subset of infants born ≤30 weeks gestation. Deficits were calculated by the difference between protein intake and presumed requirements of 3 g/kg/day and Embleton estimated that by the end of the fifth week, infants born ≤30 weeks had accrued a protein deficit of 23 ± 12 g/kg. As the current estimated requirements are now thought to be around 4 g/kg/day, this deficit is likely to be an underestimate and would be almost double based on present thinking (26). Data shown from Ernst *et al* (30) and Cormack *et al* (34) are for ELBW infants and the data shown for Radmacher *et al* are ELBW infants who were defined as having extrauterine growth retardation (92). The study by Cormack *et al* demonstrates that requirements for these infants *can* be met with an aggressive feeding protocol. However, intakes do not meet requirements until the second week of life and then remain at the lower end of the range where any catch up of the total deficit is unlikely (34).

It is evident from **Figure 1-2** that protein intakes from all studies are well below requirements in the first couple of postnatal weeks, corresponding to the lag time when enteral feeds are being established. However, even when feeds are well established, intakes remain well below the lower level of requirements in all but the Cormack study from New Zealand (34).

Data from an audit of total enteral and parenteral nutrition intake of infants <33 weeks gestation in our own neonatal unit (33) show similar results to the New

Zealand audit (34) **(Figure 1-3).** After the initial lag time during which enteral feeding is established, median intakes approach requirements but at least 25% of infants fail to meet protein requirements for the majority of the hospital admission.

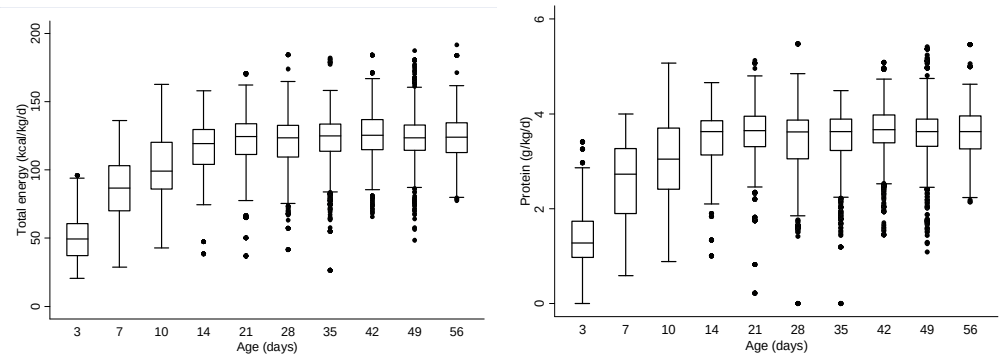


Figure 1-3: Energy and protein intakes for infants <33 wk gestation
Horizontal lines within boxes represent the median values, boxes represent interquartile ranges, and whiskers represent minima and maxima except where outliers are present, where they represent 1.5 x interquartile range.

Clearly current feeding practices fail to meet nutrient requirements for very preterm infants, particularly protein. Some neonatal units such as WCH in Australia (33) and New Zealand (34) achieve better intakes than most, but still fail to fully meet requirements. It is worthwhile examining these practices to identify possible opportunities for improving the protein intake.

1.2.4 Current feeding practices

Feeding practices vary widely between neonatal units and there are few clinical practice guidelines for the nutritional management of premature infants (93-96). Most neonatal units use either EBM or formula, either term or preterm, as a trophic (minimal) enteral feed as soon as the infant is medically stable. A Cochrane review of trophic feeding shows that this is an effective technique to reduce the number of days on parenteral nutrition, the days to reach full enteral feeds and length of hospital stay (97). In an audit of infants <33 weeks gestation

in the WCH neonatal unit, feeds were introduced at a median of day 3 (interquartile range 2-4) (33). There is widespread agreement that human milk is the feed of choice for premature infants (98, 99) and many units, including WCH, start with unfortified human milk initially. The benefits of human milk include improved host defence, gastrointestinal function, bioavailability of nutrients (98), lower rates of necrotising enterocolitis (8) and better neurodevelopment (100). However, in very preterm infants, feeding breast milk on its own is associated with poorer rates of growth (70, 82, 101-104) and bone mineralization (76, 105-107). Supplementation of human milk is therefore necessary and is now standard clinical practice to overcome this problem (68, 108, 109). As previously discussed in **Section 1.2.2.1**, a Cochrane Review concluded that multi-component fortification of human milk in preterm infants results in short term improvements in weight gain, linear and head growth with no known deleterious effect and that further studies should be directed towards the optimal composition of fortifiers rather than comparing fortification vs non fortification (109). However, there is a lack of evidence regarding the optimal time to introduce human milk fortifier (HMF), with clinical practice guidelines varying between initiating fortification at 50 – 80 mL/kg/day (96) and 150 mL/kg/day (95). The policy of the Neonatal Unit of the WCH is to fortify human milk feeds at 80 – 100 mL/kg/day.

Commercially available HMFs provide approximately 0.7 – 1.1 g extra protein per 100 mL of expressed breast milk as well as bone minerals (calcium and phosphorous), vitamins, trace elements, and some carbohydrate or fat. When commercial HMFs appeared on the market in the 1980s, manufacturers assumed an average protein composition of human milk of approximately 1.6 g/100mL, so

that when HMF providing 0.8 g protein was added, the resultant mix contained 2.4g/100 mL. If this is then fed at 150 mL/kg, it would provide the infant with a protein intake of 3.6 g/kg/day – approximately equivalent to the assumed requirement at that time. As protein requirements have been revised upwards over the years, there has been a trend for the protein content of reformulated HMFs to be increased. However, there is a general consensus amongst neonatologists that the protein content of HMFs is still inadequate (110) and this may in part be due to the assumptions made about the protein content of the expressed breast milk (EBM).

1.2.5 Protein content of human milk

The protein content of milk from mothers who deliver preterm (henceforth called preterm milk) is known to be higher than milk from mothers who deliver term infants (term milk) (111-120). This phenomenon was first described by Atkinson *et al* in 1978 (111) and then followed by a number of studies in the 1980's in the context of the nutritional adequacy of human milk for preterm infants. Interpretation of the reported protein levels in these studies has been complicated by the different techniques used to measure protein, and differing sampling of the milk. Several studies have reported total nitrogen or protein content of human milk in preterm infants using the Kjeldahl method and reported either total nitrogen or used the general conversion factor of 6.25 or the factor specific for milk, 6.38 to convert to total protein (111, 112, 114-116, 119-123). Notable from these studies is the large variation between mothers in the protein concentration in the milk with levels in the first few days postpartum varying between 44 g/L and 10 g/L (123). For ease of comparison these studies have been converted to total protein using a nitrogen conversion factor of 6.38 and summarised in **Figure 1-4**.

Values for the average protein concentration of term milk are from Jenkins, 'The Handbook of Milk Composition' page 352 (124).

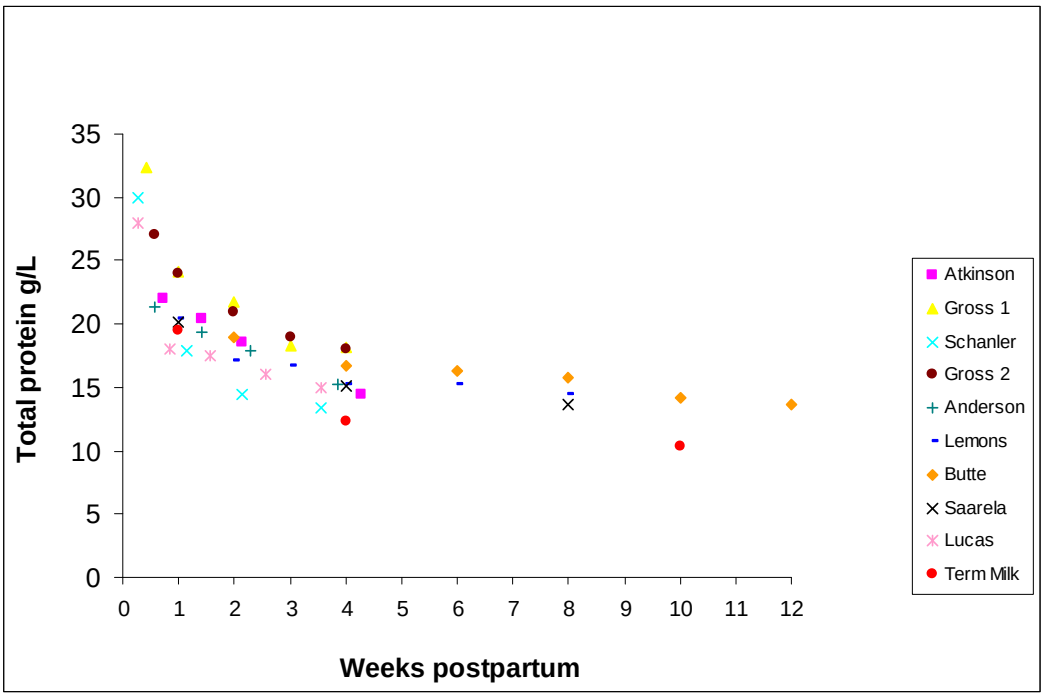


Figure 1-4. Summary of studies measuring total protein content of preterm milk
Protein = nitrogen x 6.38. Legend identifies primary author (112, 114-116, 119-121, 123, 125)

Figure 1-4 demonstrates that there is a rapid drop in protein concentration over the first few weeks, followed by a more gradual decline such that levels of preterm milk approach term milk levels by approximately three months of lactation.

Current feeding regimes usually commence with EBM and grade up the volume before fortification is added, creating a time when a nutrient deficit accrues. In the WCH audit, the median day for commencement of enteral feeds for infants <28 weeks gestation was 4 (interquartile range 3-5) and for fortification was 14 (range 8 – 40) days.

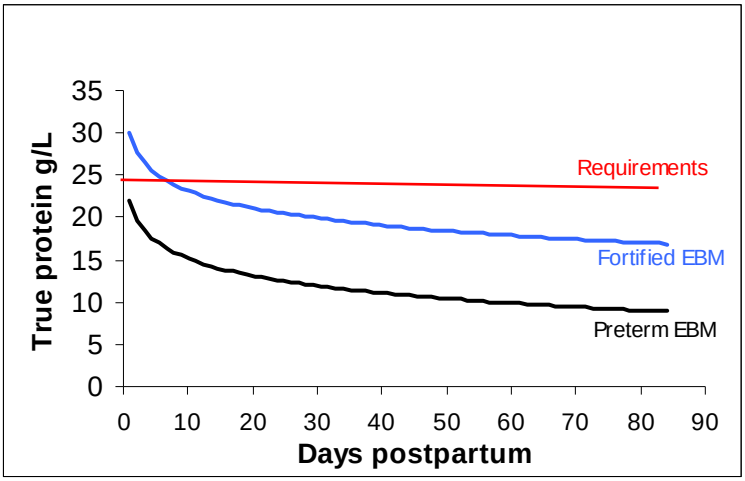


Figure 1-5 Protein content of EBM & fortified EBM over time vs requirements
Black curve shows average 'true protein' content of EBM. Blue line is with additional 8g/L from HMF. Red line is protein content needed to meet requirements when fed at 150 - 180 mL/kg

Figure 1-5 demonstrates the protein content of fortified EBM over time. The black line represents the average *true* protein content of preterm milk, derived from **Figure 1-4** and assuming that non protein nitrogen accounts for 22.5% of total nitrogen (124). The blue line shows the protein content of milk fortified with a typical commercial HMF containing 8 g protein/L. The red line is the protein level of the feed estimated to meet requirements if fed at rates between 150 and 180 mL/kg. This graph demonstrates that average intakes exceeding requirements could theoretically be achieved in the first week of life. However, fortified milk is rarely introduced to the infant this early as it corresponds to the transition phase of feeding. Once fortified feeds are established the protein level has dropped to less than that required to meet requirements and remains below requirements for the bulk of the admission. Most neonatal units do not have the facilities to routinely test the protein content of EBM and, while being aware of the limitations of current fortification regimes, they do not account for this changing protein content of the feed over time. It is therefore not surprising that growth is restricted as we

are clearly failing to meet the protein requirements of these infants for most of their hospital stay.

1.2.6 Individualised fortification regimes

Some investigators have attempted to address the problems arising from using a standard amount of fortifier by individually tailoring the amount of fortifier added either by first testing the protein content of the milk (126) or by using a biochemical marker of protein metabolism (127, 128). Polberger *et al* were the first to do this in Sweden in 1995 (126) as they had access to a rapid, accurate and cheap method of analysing milk using infra-red spectroscopy. Aliquots of EBM were analysed once a week and the amount of fortifier added was adjusted to achieve a target protein intake of 3.5 g/kg/day. Protein analysis of the milk proved to be a useful tool for individualized feeding. However, actual intakes were approximately 10% lower than the goal due to the decline in protein content of the milk over the time and the delay between sampling of the EBM and obtaining results. Polberger was concerned about both over and under supply of protein with fixed fortification regimes but unfortunately did not report on the frequency of needing more or less fortifier than usual to meet requirements.

Two other studies used an adjustable fortification regime based on biochemical markers of protein utilization (127, 128). In the first of these studies, Moro *et al* compared three groups: a human milk based fortifier added in a fixed amount (n = 12), a bovine fortifier added in a fixed amount (n = 12), and the same bovine fortifier added in amounts adjusted on the basis of twice weekly corrected serum urea nitrogen (CSUN) measurements, (n = 12). The HMF was predominantly protein with calcium, phosphorous and electrolytes added (no vitamins) and was

lower in both protein and energy compared to the bovine fortifier. To compensate, this was fed at 180 mL/kg/day whereas the bovine fortifier was fed at 160 mL/kg/day in both the fixed and adjusted regimes. Protein and energy intakes of the two fixed regimes were not different. Correction of the serum urea nitrogen (SUN) level took into account the serum creatinine level, therefore accounting for renal function. A normal CSUN was defined as 9.1 to 12 mg/100 mL: if levels were lower than this, three increments of increased fortification were defined; similarly, if CSUN levels were greater than normal, fortification was decreased in three steps. Positive rather than negative fortification levels were used most of the time during the study. Mean fortification levels used were +1.54 and +1.79 during weeks 1 and 2, respectively. Negative levels of fortification were used in the third week for 3 infants only. Note that the correction used resulted in CSUN levels that were lower than SUN levels so that fortification levels were probably underestimated. Growth was similar between the two fixed regime groups (human versus bovine protein fortifier) with similar protein and energy intakes in these groups. The adjusted regime group had higher intakes of both energy and protein but the difference only reached statistical significance for protein intake in week 2 ($p < 0.01$). Weight gain (g/day) was higher with the adjusted regime compared with both fixed regimes but only reached statistical significance when compared with the human milk protein fixed group ($p < 0.05$) (128).

A similar study by the same group in 2006 (127), compared a bovine fortifier added in either fixed (n = 16) or adjusted (n = 16) amounts, based on twice weekly BUN. In this study, the incremental fortification levels used an increased amount of fortifier for level 1 plus some supplemental protein for levels 2 and 3. Mean

fortification in the adjusted regime group were +0.9, +1.7 and +2.3 levels for weeks 1, 2 and 3, respectively. Only 1 infant required a -1 level of fortification for 3 days and then progressed to positive levels of fortification. As expected, the adjusted regime resulted in significantly higher protein intakes ($p \leq 0.05$ for weeks 2 and 3) and higher, although not significantly, energy intakes in week 3. Significantly higher weight gain ($p < 0.01$) and head circumference gains ($p < 0.05$) were found in the adjusted compared with the fixed group. Length gains were higher in the adjusted group but did not reach statistical significance. The authors found a significant correlation with protein intake and both weight gain (g/kg/day) ($r = 0.392$, $p = 0.027$) and head circumference gain ($r = 0.389$, $p = 0.029$) and no effect of energy on growth.

These studies investigating individualized regimes of fortification, evolved from concerns that adding a fixed amount of fortifier to the unknown and highly variable protein content of human milk would put vulnerable infants at risk of both protein overload and protein under nutrition. The latter two studies, however, demonstrate that protein overload is much less of an issue than protein under nutrition. They also point strongly to the role of protein in growth, despite their small sample size. However, when additional fortifier is added, all nutrients carried in the fortifier are increased, including energy, vitamins and minerals. It is therefore difficult to know if the improved growth is due to protein alone, or a cocktail effect of increasing a number of nutrients. In order to investigate the direct effect of protein on growth, it is necessary to examine trials that have compared different levels of protein while keeping constant other nutrients known

to affect growth. The next section appraises the literature comparing human milk fortified with differing protein concentrations and the effects on growth.

1.3 Randomised controlled trials comparing human milk fortifiers with differing levels of protein

In this section randomised trials which have compared the efficacy of two human milk fortifiers with growth as their primary outcome will be systematically reviewed using the Cochrane Collaboration method. The aim of this review is to evaluate the effect of feeding an increased protein HMF with a reference HMF on the rate of growth (weight, length and head circumference), safety and feeding tolerance. Subgroup analyses of trials which have kept the energy content constant were planned *a priori.*

1.3.1 Methods

The systematic review was undertaken using the standard method of the Cochrane Collaboration (129) and the Neonatal Collaborative Review Group as outlined in the Cochrane Library.

1.3.2 Search Strategy

Computerised searches were conducted of the Cochrane Central Register of Controlled Trials (CENTRAL, The Cochrane Library, (January 2010 issue), MEDLINE (1966 – January 2010), CINAHL (1982 – January 2010) and EBMASE (1980 – January 2010). The Medical Subject Headings (MeSH) used included: milk, human; infant, newborn; infant, newborn, diseases; food, fortified; protein, dietary. 'Human milk fortifier' was used as a text search term. Results were restricted to 'clinical trial' or 'randomised controlled trial'. No language

restrictions were applied and reference lists from identified articles were searched for other potential articles.

1.3.3 Criteria for selecting studies

All trials enrolling preterm infants (<37 weeks gestation) who received human milk with added HMF, either exclusively or supplemented with formula were considered. The intervention must have compared two or more protein concentrations in HMF. Trials must have reported one or more of the outcomes of interest: rate of growth (rate of gain for weight, length or head circumference), safety (biochemical markers of protein metabolism and excretion, adverse events) and tolerance (gastrointestinal problems).

1.3.4 Assessment of methodological quality

Included trials were assessed for quality using the following headings from the Cochrane risk of bias table (129)

- Sequence generation
- Allocation concealment
- Blinding
- Completeness of outcome data

1.3.5 Data synthesis and analysis

The mean difference in growth rate (weight gain in g/kg/day, length and head circumference gain in cm/week) of preterm infants fed lower versus higher protein fortifier was computed in the RevMan programme (Review Manager version 5.0.10) using the fixed effects model. The random effects model was used when heterogeneity was substantial as measured by having an I^2 statistic >50% (129).

Subgroup analyses were planned *a priori* for those trials that varied the protein content of the fortifiers while keeping the energy constant.

1.3.6 Results

One hundred and fifteen papers were identified. The majority of these (n = 78) were excluded as either they did not relate to fortified EBM or they compared breast milk with formula. Thirty seven papers were considered in more detail.

1.3.6.1 Excluded papers

Excluded studies are detailed in **Table 1-3.** The three papers comparing individualised fortification regimes (127, 128, 130) have been discussed in more detail in **Section 1.2.6**.

Table 1-3 Characteristics of excluded studies

Study	Reason for exclusion
Mondalou 1986 (66) Kashyap 1990 (70) Lucas 1996 (72) Nichol 1999(74) Gathwala 2007 (131) Mukhopadhy 2007(132)	Compared unfortified with fortified human milk with growth as primary outcome
Ronnholm 1982 (62) Boehm 1988 (65) Polberger 1990 (133)	Compared unfortified with fortified human milk with protein metabolites (urea, amino acids) as primary outcome
Gathwala 2008 (134)	Compared unfortified with fortified human milk with gastric emptying as primary outcome
O'Connor 2008 (135)	Compared unfortified with fortified human milk post discharge with growth as primary outcome and lactation success as secondary outcome
Morley 2000 (53)	Long term follow up of earlier trial (Lucas 1996 (72))
Pettifor 1989 (69) Wauben 1998 (73) Faerk 2000 (75) Schanler 1995 (136)	Primary outcomes were bone mineral density
Berseth 2004 (137)	Compared two different fortifiers with similar protein content but differing iron content
Wauben 1999 (138)	Compared a multi-nutrient fortifier containing zinc with calcium and phosphorous fortification
Noack 1991 (139) Doege 2007 (140)	Compared fortified human milk with preterm formula
Polberger 1989 (64) Ronnholm 1986 (108) Ronnholm 1984 (141)	Compared EBM fortified with either fat or protein
Boehm 1990 (142)	Compare protein quality from different sources rather than quantity Compared human milk derived fortifier with meat hydrolysate Compared bovine fortifier with human albumin
Boehm 1991 (143) Boehm 1993 (144) Hagelberg 1990 (145) Moro 1991 (146)	Compared human milk derived fortifier with bovine Compared human milk derived fortifier with bovine Compared human milk derived fortifier with bovine
Arslanoglu 2006 (127) Moro 1995 (128) Polberger 1995 (130)	Compared individualised fortification regimes
Arslanoglu 2009 (147)	Not a clinical trial. Re-analysis of data from an earlier trial (127) focusing on protein intake

1.3.6.2 Included papers

Five papers reporting 5 separate studies were included in this review (128, 146, 148-152) and these are summarised in **Table 1-4**.

1.3.6.2.1 Participants

All trials restricted enrolment to relatively healthy preterm infants, without major congenital or clinical problems, but the study populations varied in gestational age and birth weights. An upper limit of birth weight (BW) was specified for all studies. Zuppa *et al (152)* and Porcelli *et al (151)* used a BW upper limit of 1500 g. Sankaran *et al* (149) and Reis *et al* (150) used 1600 g and Metcalf *et al* (148) used 1800 g. Both Porcelli *et al* and Sankaran *et al* also used a lower limit of BW, which was 600 g in both studies. Three studies restricted enrolment to AGA infants (148, 151, 152). An upper limit of gestational age (GA) was also defined in all studies with the exception of Zuppa *et al (152)*. Metcalf *et al* (148) and Sankaran *et al* (149) used an upper limit of 34 weeks gestation. Reis *et al* (150) used 33 weeks and Porcelli *et al* (151) use 32 weeks. Both Porcelli *et al* and Sankaran *et al* also defined a lower GA limit of 25 and 24 weeks, respectively. While Zuppa *et al* (152) did not state any limits for GA, the range of GA reported was 29 to 34 weeks, making the infants in this study more mature than infant populations in the other studies.

1.3.6.2.2 Interventions

Details of the interventions used and the study duration can be found in **Table 1-4.** The interventions varied in several respects including the dose of protein, hydrolysed versus intact protein (148), liquid versus powdered fortifier (149) and

additives such as fat plus emulsifier (150), vitamins, minerals and trace elements . Metcalf *et al* (148) and Zuppa *et al* (152) also included a third arm of infants fed formula to their studies. The groups fed infant formula have not been included in this review.

Study duration varied considerably with little consistency of definition for both the starting and end points of the study. The studies usually commenced once a predefined enteral intake was reached but this varied between 100 (150) and 150 mL/kg/day (151). Metcalf's *et al* study commenced once full enteral feeds were reached but 'full' was not defined (148) and Zuppa did not define the study start (152). Three studies ceased fortification when the infant reached 2 kg in weight (148, 149, 152). Other studies defined the end as discharge or study day 29 (150) or when weaned to unsupplemented milk (151).

1.3.6.2.3 Outcomes

All studies reported growth for the study duration. Weight gain in g/kg/day was reported for all studies except Sankaran *et al* (149), who reported it in g/day. Length gain was not reported by Metcalf *et al* as the measures were found to be unreliable (148) but all other studies reported length gain in cm/week. All studies reported head circumference gains in cm/week. Reis *et al* (150) was the only study to state that trained personnel and standardised procedures were used for the growth measurements. Porcelli *et al* (151) stated that standardised equipment was used across sites for growth measurements but did not mention training or procedures. None of the other studies gave details about the training, procedures or equipment for growth measurements.

Three studies examined some biochemical indicator of protein metabolism namely SUN or BUN (150, 151), albumin and total protein (149).

Confirmed or suspected NEC was reported in 3 studies (148, 150, 151). Some measure of feeding tolerance was reported in all studies except Zuppa *et al* (152) and these included gastric residue, incidence of vomiting and diarrhoea and the number of times that feeds were withheld.

1.3.6.2.4 Trial quality

All studies in this review used randomisation to allocate the treatment. Two studies were small with between 10 and 14 infants in each group (148, 152).

Sequence generation, allocation concealment and blinding

Sankaran *et al* (149) was the only study to describe the method of sequence generation which was computer generated blocks. None of the studies stated whether there was adequate concealment of the allocation. Blinding was only reported in Reis *et al* (150), as in most of the other studies, the trial products were not identical and so blinding was difficult to achieve. Porcelli *et al* stated that the study was not double blinded due to different mixing requirements of the fortifiers but that the investigators responsible for the outcome assessments were blinded to the allocation (151).

Completeness of outcome data

Zuppa *et al* was the only study to report outcome data for all infants enrolled (152). This trial enrolled 10 infants in each arm and did not report any attrition or exclusions. None of the remaining studies reported intention to treat analyses according to the Cochrane definition: ‘All participants are included in the arm to

which they were allocated, whether or not they received (or completed) the intervention given to that arm' (129). Reis *et al* (150) reported data for 'intention to treat' analyses but in fact, this was defined as all infants who reached study day one. Twenty five infants were enrolled but did not reach study day 1 and this attrition was explained in only 13 of these infants who never received a fortified feed. Reis *et al* stated an *a priori* analysis of those infants who complied with the protocol and results were reported for all infants who reached study day 1 and the 'per protocol' subgroup (150). Porcelli *et al* enrolled 90 infants and assessed 64 (151). Data were stated to be analysed in two populations, intention to treat and the 'evaluable' population. Although it was stated that the intention to treat analyses confirmed the 'evaluable' population analyses, data were only shown for the 64 'evaluable' infants. Reasons for the 26 infants discontinuing the study were provided and these included formula intolerance and protocol violations. Metcalf *et al* withdrew one infant from the control group because of NEC (148) but did not state the number of infants assessed for each outcome measure. Sankaran enrolled 60 infants and assessed 41 (149). Eleven infants were withdrawn because of clinical problems and the others were removed because of early discharge from hospital. It would appear that Sankaran used *post facto* selection as infants were included if their mother was able to supply ≥90% of their daily requirements.

Overall, the lack of blinding, unclear reporting of the randomisation procedures and incomplete data assessment may introduce random error or bias into these studies.

Table 1-4 Summary of trials included in meta-analyses of higher vs lower protein fortifier for promoting growth in preterm infants

Reference		**Metcalf 1994 (148)**	**Sankaran 1996 (149)**	**Porcelli 2000 (151)**	**Reis 2000 (150)**	**Zuppa 2004 (152)**
Stated aim of study		Compare efficacy of two commercial bovine HMFs and a preterm formula	Evaluate the added nutritional value of two commercial HMF (liquid and a powder)	Compare a new human milk fortifier containing more protein than a reference one	Evaluate growth and nutritional status of infants receiving newly formulated HMF with a commercial HMF	Compare LBW infants fed with preterm formula with two other groups fed different fortifiers
Participants		AGA preterm infants GA < 34 weeks BW: < 1800 g	Healthy preterm infants GA: 24-34 weeks BW: 600-1600 g	AGA VLBW infant GA: 25-32 weeks BW: 600-1500 g	Preterm infants GA: <= 33 weeks BW: <= 1600 g	AGA preterm infants GA: not specified BW: < 1500 g
Fortifier composition	Ptn (g) & E (Kcal) added to 100mL EBM	Protein Energy	Protein Energy	Protein Energy	Protein Energy	Protein Energy
	Intervention	+ 0.9 + 18	+0.7 +14	+ 1 + 13	+ 0.9 + 12	+0.8 +15[b]
	Control	+ 0.7 + 14	+0.3 +7[a]	+ 0.7 + 14	+ 0.6 + 12	+0.7 +14
Other differences						
Intervention		Hydrolysed ptn, no vits (Nestlé FM-85)	Powder, + CHO, vits & mins (Mead Johnsons Enfamil)	100% whey based , CHO vits, min (Wyeth, experimental)	Whey based + fat, emulsifier (Ross, Similac powder)	Milupa, Eoprotin
Control		Intact ptn, + vits (Mead Johnsons Enfamil)	Liquid mixed 1:1 with EBM, fat, CHO, vit (Ross, Similac liquid)	Whey: casein 60:40, CHO, vit, mins (Mead Johnsons Enfamil)	Whey:casein, no fat, (Mead Johnsons Enfamil)	Mead Johnsons Enfamil
Study duration			3 consec days of	When HMF intro	Full strength	Not defined
Start		Tolerance of full feeds	120 mL/kg tolerance		fortification and enteral intake >100 mL/kg	
End		Weight of 2 kg	2 kg or ave wt gain 35 g/day over 10 days	When weaned to unsupplemented milk	Study day 29 or D/C	Weight of 2 kg
Infants	Enrolled	$n_i = 14$ $n_c = 11$	$n_i = 31$ $n_c = 29$	$n_i = 47$ $n_c = 43$	$n_i = 74$ $n_c = 70$	$n_i = 10$ $n_c = 10$
	Assessed	$n_i = 14$ $n_c = 10$	$n_i = 19$ $n_c = 22$	$n_i = 35$ $n_c = 29$	$n_i = 60$ $n_c = 51$	$n_i = 10$ $n_c = 10$
Trial quality	Sequence generation	Unclear	Adequate	Unclear	Unclear	Unclear
	Allocation concealment	Unclear	Unclear	Unclear	Unclear	Unclear
	Blinding	No	No	Investigators blinded	Yes	Unclear
	Incomplete outcome data	n_c 1/11	n_i 12/31 n_c 7/29	n_i 12/47 n_c 14/43	n_i 14/74 n_c 19/70	No drop outs

[a]Computed from (149) Table 4, Pg 1148; [b] Computed from (152) Table 2, pg 46 and manufacturer's information; Ptn = protein E = energy CHO = carbohydrate vits = vitamins mins = minerals wt = weight
n_i = number of infants in intervention group; n_c = number of infants in control group

1.3.7 Meta-analyses of included studies (Table 1-4)

1.3.7.1 Growth outcomes

1.3.7.1.1 Weight gain

Studies reporting weight gain over the study period as g/kg/day are included in this analysis (148, 150-152). Sankaran *et al* (149) reported weight as g/day and so was excluded. Porcelli *et al* (151) reported standard errors (SE) and these were converted to standard deviations (SD) by multiplying by the square root of n, the sample size. There is substantial heterogeneity (I^2 80%) and a random effects model was used. However, the heterogeneity may indicate that studies could not be combined. Indeed, the diversity between studies was both clinical (variations in participants, interventions and outcomes) and methodological (variability in study design) and is discussed in more detail in **Section 1.3.6.2**. The difference in protein content of the fortifiers compared in Zuppa *et al* (152) was only 0.1 g and may be too small to translate to a difference in protein intake. Other differences in the intervention include intact versus hydrolysed protein and the addition of other components e.g. vitamins, trace elements, fat and emulsifier. Trial methodology also varied with only two of the four studies blinded (150, 151) and sequence generation and allocation concealment unclear for all studies.

Porcelli *et al* (151) and Reis *et al* (150) reported a statistically significant increase in weight gain with the higher protein fortifier whereas Zuppa *et al* (152) and Metcalf *et al* (148) found a non-significant difference. The overall meta-analysis did not reveal a significant difference in weight gain with higher protein fortifier (WMD 1.16 g/kg/day, 95% CI -0.92 to 3.24, $p = 0.27$, $n = 227$) (**Figure 1-6**). The

subgroup analysis of isocaloric trials showed a similar non-significant difference in weight gain (WMD 1.36 g/kg/day, 95% CI -1.51 to 4.22, p = 0.36, n = 203).

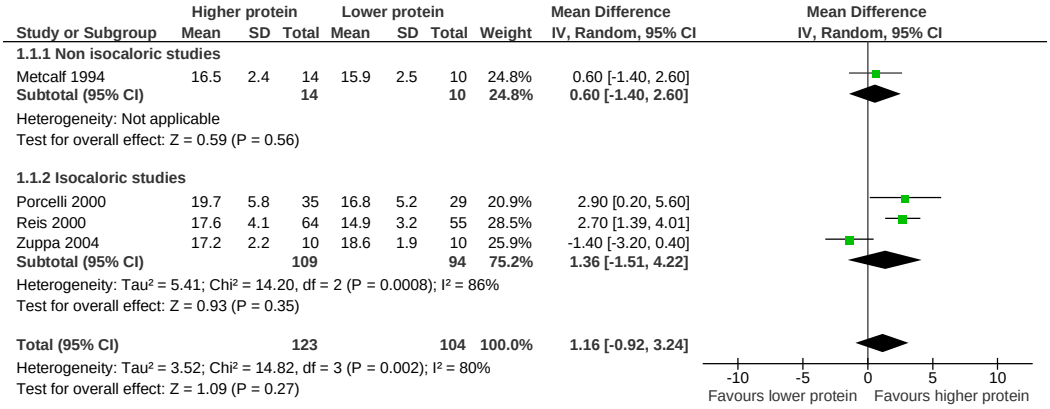


Figure 1-6 Meta-analysis forest plot showing mean difference in weight gain (g/kg/day) for preterm infants fed HMF with higher versus lower protein content
IV = inverse variance; CI = confidence interval

1.3.7.1.2 Length gain

Four of the five studies reported length gain in cm/week over the study period and were included in this analysis (149-152). Sankaran *et al* (149) reported mean ± SE and this was converted to the SD by multiplying by the square root of n, the sample size. The resulting SD for the intervention is considerably larger than either the standard protein group or SDs reported in the other studies. It is not clear why this is the case. Metcalf *et al* deemed their growth measurements to be unreliable and did not report them (148).

A fixed effect analysis was used as there is no heterogeneity noted from the I^2 statistic. This is despite the fact that one of the studies in this analysis compared a liquid with a powdered fortifier (149) and so differed significantly from other studies which compared two powdered fortifiers. Reis *et al* found a significantly higher rate of length gain in the higher protein group (150) whereas all other

studies found a non-significant difference (149, 151, 152). Infants receiving the higher protein fortifier had a small, significant increase in length gain of 0.12 cm/week (95% CI 0.02 to 0.22, p <0.01, n = 237) (**Figure 1-7**). The subgroup of isocaloric trials showed a similar result with a significant increase in length gain of 0.12 cm/week (95% CI 0.03 to 0.22, p <0.01, n = 196).

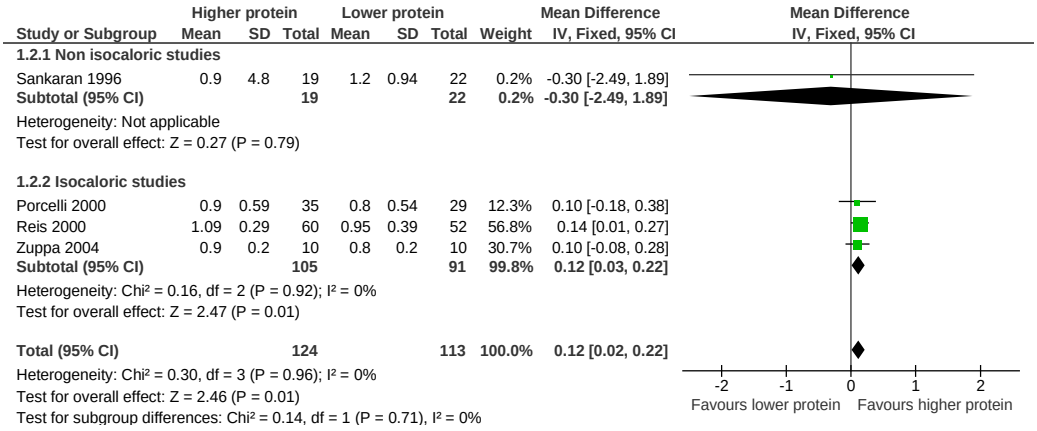


Figure 1-7 Meta-analysis forest plot showing mean difference in length gain (cm/week) for preterm infants fed HMF with higher versus lower protein content
IV = inverse variance; CI = confidence interval

1.3.7.1.3 Head circumference gain

All five studies reported head circumference gain in cm/week over the study period and were included in the analysis (148-152). Porcelli *et al* (151) was the only study to find a statistically significant increase in head growth with higher protein fortifier, while all other trials found a non-significant difference. Substantial heterogeneity of the treatment effect is observed for studies overall (I^2 69%) and also for each subgroup (I^2 62% for non-isocaloric and 75% for isocaloric studies) and a random effects model was used. The study diversity has been discussed previously in **Section 1.3.6.2**. Greater heterogeneity is evident in the subgroup of isocaloric trials (I^2 75%) and this may be due to Zuppa's small

study (n = 10 in each group) with only a 0.1 g difference in protein content of the fortifiers which may not be enough to demonstrate a difference in head growth. The overall results show no difference of higher protein fortifier on head growth (WMD -0.04, 95% CI -0.22 to 0.14. p = 0.63, n = 260) (**Figure 1-8**). The subgroup analysis of isocaloric trials shows a similar non significant difference in head growth (WMD -0.01, 95% CI -0.23 to 0.26. p = 0.91, n = 196).

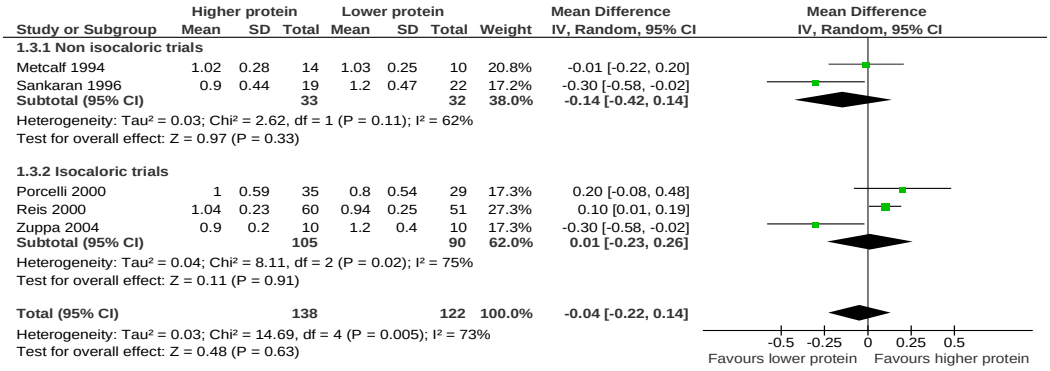


Figure 1-8 Meta-analysis forest plot showing mean difference in head circumference gain (cm/week) for preterm infants fed HMF with high versus low protein content
IV = inverse variance; CI = confidence interval

1.3.7.2 Safety outcomes: Biochemical variables

Urea nitrogen is an end product of amino acid metabolism and its level is affected by dietary protein intake, hydration status and renal function. It is often used as a marker of protein excess but in preterm infants it is also a valuable indicator of protein intake as it parallels dietary intake and responds rapidly to changes in intake (153-155). It is also a readily available and commonly used test in a clinical setting, making it an ideal metabolic indicator of protein status. Only one trial (151) reported BUN levels. Porcelli *et al* compared mean BUN between groups both at baseline and study end. Baseline levels did not differ, however

mean levels at study end were significantly greater in the higher compared with lower protein group (2.1 SE 0.2 and 1.7 SE 0.1; $p = 0.04$, respectively). Despite being higher, levels were within the accepted range and therefore the clinical significance of this is unclear.

Serum albumin is a commonly used indicator of protein nutritional status. However because of its long half life (about 20 days) it is best used to assess longer term changes in nutritional progress. Only one study (149) assessed serum albumin and did not find a significant difference between groups either at study entry or exit.

Total protein

Total protein is also used as an indicator of protein nutritional status. Sankaran *et al* was the only study to measure total protein at study entry and exit and found no differences between groups (149).

Amino acids

Pathways for amino acid metabolism are immature in preterm infants and they are therefore susceptible to both inadequate and excessive amino acid levels. Some amino acids (eg phenylalanine, tyrosine) are neurotoxic and high dietary protein intakes can result in symptoms of lethargy and poor feeding and occasionally lead to neuro-developmental problems if levels are extreme (88, 156). Plasma amino acids levels are indicative of protein quality as well as protein metabolism. Therefore studies that have compared hydrolysed with intact protein, or proteins with differing whey to casein ratio may expect to see some difference in the blood

amino acid profile of the infant. Despite this, no trials in this review measured amino acid levels.

1.3.7.3 Safety outcomes: Adverse events

Various measures of adverse events were reported in three out of the five studies (148, 150, 151). Metcalf *et al* (148) reported one incidence of NEC (in the higher protein group) from 24 infants and this infant was withdrawn from the study. Porcelli *et al* (151) used the same definition as the Federal Drug Administration for a study event i.e. any untoward medical occurrence regardless of whether the event was believed to have a causal relationship to the human milk fortifier. They found no difference between groups in the overall incidence. Reis *et al* (150) documented suspected NEC (3 from 60 in the higher protein group and 8 out of 51 in the control group). There was only one case of confirmed NEC and this was in the control group. The number of infants requiring oxygen, ventilation, steroids, or having apnea or bradycardia was not different between groups.

1.3.7.4 Tolerance outcomes

Various measures of tolerance were reported in four out of the five studies and no differences were found between groups (148-151). Metcalf *et al* (148) noted that the number of infants who had feeds stopped (4 of 14 in high protein group and 5 of 10 in the control group) was not different between groups but considered this a high rate of intolerance when compared to the third arm of the study which was infants fed preterm formula who showed no feeding intolerance. Sankaran *et al*

(149) reported gastric residue, abdominal girth, diarrhoea and vomiting and found no differences between groups. Porcelli *et al* (151) also documented diarrhoea, vomiting and gastric residuals and found no differences between groups. Similarly, Reis *et al* (150) found no differences between groups with respect to the number of infants having feeds withheld for greater than a day (5 of 69 in high protein group and 8 of 62 in control group), gastric residuals greater than 5 mL, abdominal distension or emesis.

1.3.8 Discussion

The meta-analyses have indicated a small advantage of high protein fortification for length gain but no overall advantage for weight gain or head circumference growth. The order of magnitude of the increase is the same as that seen in the Cochrane review comparing multi-component fortification of milk with no fortification (61) but less than that found in the review comparing protein fortification with no fortification (58). This smaller effect size may be because unfortified human milk does not meet requirements for preterm infants (82, 102, 103), so the addition of any macronutrient supplement will move the dietary intake closer to requirements. In contrast, the meta-analyses done here compare different levels of fortification, some of which may already meet requirements for some infants, and are focused on finding the optimal protein fortification.

However, data from the meta-analyses presented here should be interpreted with caution. There is some inconsistency in the results and this may reflect the variability in the fortifiers used and other differences in study design. In addition,

some studies included had small sample sizes and wide confidence intervals and the risk of bias is high in several of the studies included.

Data regarding safety outcomes were difficult to combine in a meta-analysis due to the different biochemical markers used. Urea nitrogen was only reported in one study where it increased in the higher protein group, paralleled dietary intake and stayed within the acceptable range. Albumin and total protein were also only reported in one study and no differences between groups were shown. This is not surprising given the long half life of these proteins and the short duration of the study (2 to 3 weeks).

Tolerance was also evaluated and reported in a variety of ways, making a meta-analysis difficult. However, there were no significant differences reported between groups in any of the studies regardless of what measures were used. Studies have not generally been powered to detect these differences.

Overall these studies report results for 260 infants. There are some inconsistencies in growth results particularly for weight and head circumference gains. However, higher protein fortification may provide a small advantage for length gain. While the incidence of adverse events occurring with higher protein fortification does not appear to be increased, the small number of infants in this review precludes any robust conclusions. Studies sufficiently powered to detect small differences in adverse outcomes are required.

1.4 Rationale for thesis

Breast milk is the preferred feed for preterm infants and confers significant neuro-developmental and other health benefits. However, preterm infants fed EBM (even when fortified) tend to have poorer growth than infants fed preterm formula and this seems at least partly due to the increased protein content of formula.
It is now standard practice to fortify breast milk with a multi-component supplement containing protein and other nutrients. However, the optimal protein content of the fortifier for growth has not been defined. Current protein levels in commercial fortifiers do not account for the rapid fall of protein in EBM over the first 2-4 weeks postpartum and are generally thought to be inadequate. Few studies have compared the protein content of fortifier, while keeping energy constant, and individualised fortification studies that have added enough protein to fully meet requirements have also increased total energy. Preterm infants fed EBM also have a shorter exposure to the higher protein intake that results from fortifier as they progress to direct sucking feeds during their hospital stay. This may further disadvantage them in comparison to formula fed infants.

The following chapters describe a randomised controlled trial comparing the effects of isocaloric HMF with two different protein concentrations on growth in preterm infants. The control group received fortifier with a protein content equivalent to current practice (1 g protein/100mL EBM) while the treatment group received a higher protein fortifier, providing 1.4 g protein/100mL EBM. The intervention was designed to meet requirements and account for the fall in the protein content of EBM over the first 2-3 weeks of life. The primary efficacy outcome was linear growth, measured weekly until discharge (D/C) or estimated

date of delivery (EDD), whichever came first. Weight and head circumference gains were also measured. The primary safety assessment was biochemical markers of protein metabolism. Other secondary outcomes included clinical measures of disease and adverse events.

Chapter 2 Milk Analyser

2.1 Introduction

Measurement of the macronutrient content, particularly protein, of human milk in this trial was necessary to determine changes in protein concentration. As discussed in **Section 1.2.5**, milk from mothers who deliver preterm infants is known to have a high variability in protein content and to change rapidly over the first few weeks (111, 112, 114-116, 119-123). The protein concentration of preterm milk has been reported to vary between 1.0 and 4.4 g/100 mL (123) and this variation is greater than the 0.4 g difference in protein content of the human milk fortifiers used in this study. It was therefore important to determine if the protein intervention translated to an equivalent protein difference in dietary intake. Determination of the macronutrient concentration in the milk allowed us to calculate energy intakes and, in conjunction with infant formula manufacturers' information, compute total dietary intake for the study participants.

Traditional methods for determining protein, such as Kjeldahl, require relatively large volumes of milk for laboratory analysis, often off-site, resulting in significant costs and lengthy delays before results are available. For the serial measurements done for this study we needed to develop a method that was accurate, on-site and required only a small volume.

Infra-red (IR) technology has been used in the dairy industry to measure the macronutrients of various herd milks (cow, sheep, goat and buffalo) for some time (157). More recently, IR technology has been used to analyse human milk (158,

159). The Child Nutrition Research Centre (CNRC) acquired a MilkoScan™ Minor analyser, manufactured by FOSS, Denmark, which uses IR technology. In 90 seconds, the MilkoScan™ Minor can simultaneously provide an analysis of fat, protein, lactose, solids-non-fat, total solids and freezing point depression from one 6.5 mL sample of milk. The machine is delivered pre-calibrated for various herd milks (as above).

2.2 Validation of MilkoScan™ Minor for Human Milk Macronutrient Analysis

2.2.1 Study aim

The aim of this study was to calibrate the MilkoScan™ Minor to analyse human milk and to determine the precision and accuracy of the instrument. This method was validated according to the guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), Validation of Analytical Procedures, which have been adopted by the Therapeutic Goods Administration (TGA), Australia (160).

Three tasks were performed to achieve this:

1) Evaluation of accuracy and linearity using a comparison of methods experiment for protein, fat and carbohydrate
2) Determination of two levels of precision:
    a. Repeatability (intra-assay variation) and
    b. Intermediate precision (inter-assay variation)
3) Determination of a Quantitation Limit (QL) calculated from the calibration curve.

NOTE:
This figure is included on page 161 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2-1 MilkoScan™ Minor IR Technology Adapted from Foss (161)

2.2.2 Principles of MilkoScan™ Minor

The principles of operation of the Milk Analyser are demonstrated in **Figure 2-1**(161). A sample of milk is pumped into the machine, heated and homogenised before passing into a cuvette (a 50 micron spacing between two glass plates) where it is analysed. Infra-red rays of wavelength 1 – 12 µm first pass through the sample where absorbance takes place according to the components in the sample. The rays then pass through a rotating wheel onto four optical filters and detectors. Each filter allows one specific wavelength to pass and the energy of this wavelength is proportional to the amount of the component present. The detector transforms the wavelength to an electrical signal which is then converted, using an algorithm, to give a percentage of the various components. Wavelength details are discussed below with the relevant macronutrient **(Section 2.2.5).**

2.2.3 Participants

Milk samples were collected from lactating mothers between May 2006 and August 2008. To ensure a broad range in the protein content of the milk we had 3 recruitment strategies: we recruited women whose infant was a current inpatient in the Neonatal Unit at FMC, women in the community, through the Australian Breastfeeding Association or by word of mouth. Women who were identified as having milk surplus to their infant's requirements were approached for the study. Approval for this study was granted by the Flinders Clinical Research Ethics Committee at FMC in Adelaide, South Australia. Women received both verbal information and a written information sheet about the study, and all participants signed an approved consent form.

2.2.4 Milk samples

No specific instructions were given regarding the method or timing of expressing the milk sample to ensure variation in the fat content of the specimen. Women donated a minimum of 50 mL of milk at various stages of their lactation ranging from the first week to 18 months postpartum. This variation optimised the chance of obtaining a wide range in the protein concentration of the milk. A total of 30 samples were collected from 27 women. Seven samples were from 5 women who had delivered a preterm infant (two women donated milk samples at different time points). All samples were frozen at minus 20^{o}C within 24 hours of expressing. Once all the samples were collected, they were thawed at room temperature and then placed in an incubator at 37^{o}C for 30 minutes before being aliquotted under constant agitation for later analysis. An 8 mL aliquot was immediately assessed with the MilkoScan^{TM} Minor for protein, fat and carbohydrate.

2.2.5 Linearity and Accuracy: Comparison of Methods Experiment

2.2.5.1 Protein

2.2.5.1.1 Methods: Protein

Total nitrogen and non-protein nitrogen (NPN) were determined by the Kjeldahl method and converted to protein using a factor of 6.38. Human milk contains approximately 20 – 25% NPN, a higher level than most other milks and this fraction consists of peptides, urea, uric acid, ammonia and free amino acids. It has been estimated that approximately 27% of NPN of human milk is bioavailable (127, 162). The MilkoScan^{TM} measures protein by the stretching vibration of the N-H bond within the peptide. As this N-H bond is common to both protein and peptides, the MilkoScan^{TM} Minor measures both these components but will not measure other NPN such as free amino acids. In order to correct for this we used

the method described by Arslanoglu et al (127) to calculate the reference protein for the MilkoScan™ Minor using the following equations:

True protein = (total nitrogen – NPN) x 6.38

Reference protein = 'True protein' + (27% of NPN x 6.38)

Twenty six of the 30 milk samples collected were of sufficient volume to be analysed for protein content at Dairy Technical Services Ltd., Flemington, Victoria, Australia; a National Association of Testing Authorities (NATA) accredited facility. Chilled samples were transported to their laboratory by courier within 24 hours of aliquotting. Analyses of total nitrogen and non protein nitrogen were done using the Kjeldahl method according to Australian Standards 2300.1.2.1 (163) and 2300.1.2.2.8.3 (164) respectively.

2.2.5.1.2 Results: Protein

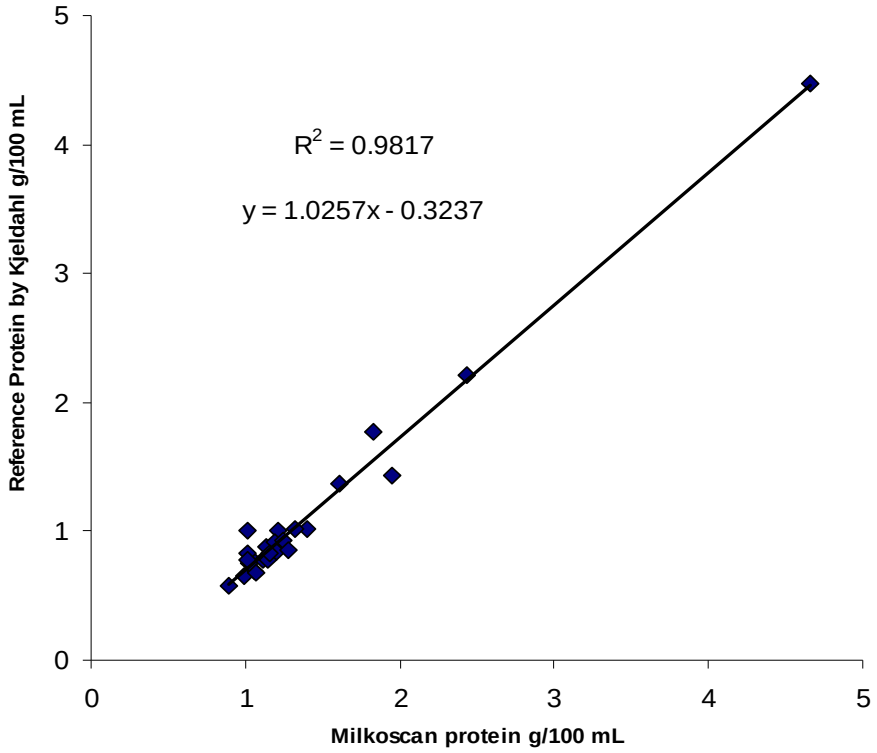


Figure 2-2 Protein: Reference (Kjeldhal) vs Measured (MilkoScan™ Minor) (n = 26)

The accuracy and linearity for protein are shown in **Figure 2-2** together with the equation and correlation coefficient. These results are across the range of 0.57 to 4.7 g protein /100 mL. The MilkoScan^{TM} Minor PC software uses the slope and intercept of the regression line and adjusts subsequent readings.

2.2.5.2 Fat

2.2.5.2.1 Method: Fat

All samples (n = 27) were analysed for fat at the Nutrition & Functional Food Research Laboratory, School of Agriculture, Food and Wine, University of Adelaide, using the Gravimetric method. Samples were analysed three times and the average of these measurements was taken as the reference fat. The MilkoScan^{TM} Minor IR absorption of fat is due to the stretching vibrations in the C-H bonds of the fatty acid chains and will therefore depend on both the size and number and fat molecules.

2.2.5.2.2 Results: Fat

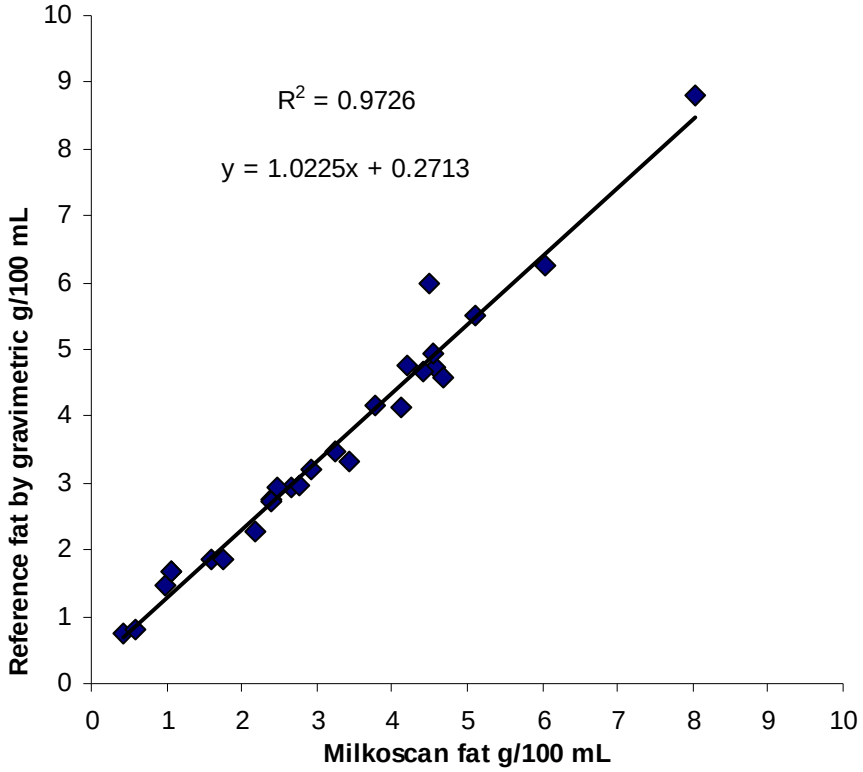


Figure 2-3 Fat: Reference (Gravimetric) vs Measured (MilkoScan ™ Minor) (n = 27)

Figure 2-3 demonstrates the accuracy and linearity of the MilkoScan™ Minor for fat across the range of 0.75 to 8.8 g/100 mL. The equation of the regression line was used to adjust the MilkoScan™ Minor.

2.2.5.3 Carbohydrate

2.2.5.3.1 Introduction

The principal carbohydrate (CHO) in human milk is lactose but oligo- and mono-saccharides are also present. Oligosaccharides have been shown to contribute between 1.2 and 1.4g/100 mL (165), a greater component than protein and therefore significant. Oligosaccharides are resistant to digestion in the small intestine and found intact in both the digestive and urinary tract, and so it is

thought that most oligosaccharides are nutritionally unavailable to the infant (166). The MilkoScan™ Minor measures CHO by the absorption of the C-OH bond which is characteristic of all CHOs and therefore will include oligosaccharides and any monosaccharides present. Our purpose for quantitating CHO was to calculate macronutrient intake and therefore energy intake; hence we wanted to calibrate the MilkoScan™ Minor for the nutritionally available CHO (lactose) in human milk. A reasonable correlation with a lactose reference measure would allow us to correct the analyser using the slope and intercept.

2.2.5.3.2 Method: Lactose analysis

We were not confident that this method would produce a good correlation so limited this experiment to ten samples, as a pilot study. The samples were analysed for lactose monohydrate at Dairy Technical Services Food Laboratories, Kensington, Victoria using the reference method, AS2300.6.6 and compared with the MilkoScan™ Minor measurement.

2.2.5.3.3 Results: Lactose analysis

One MilkoScan™ Minor measurement was discarded due to an equipment malfunction and a total of nine results were compared as shown in **Figure 2-4**.

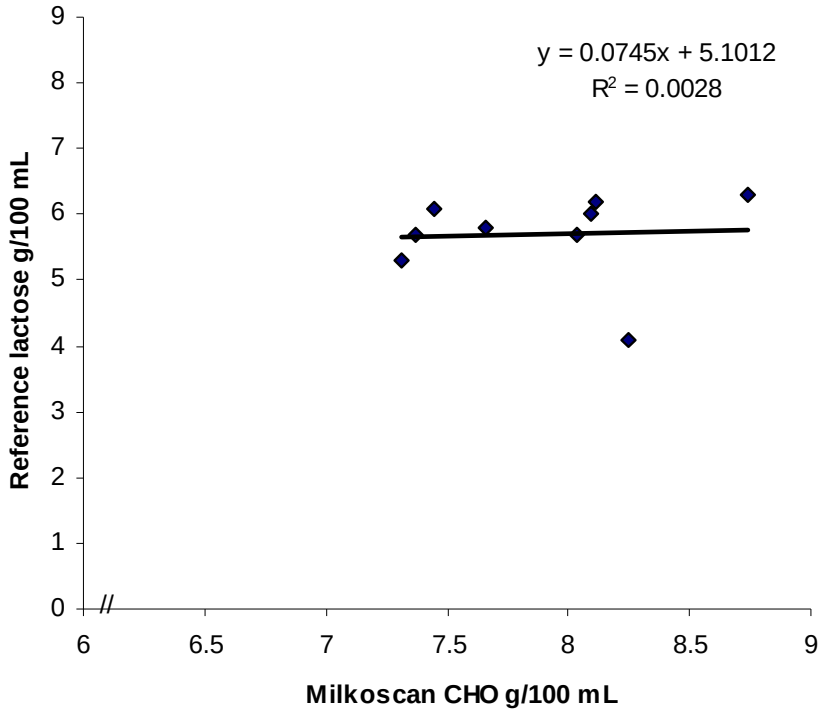


Figure 2-4 Lactose: Milkoscan vs reference (n = 9)

The R^2 was < 0.003 and the equation is shown on the graph.

2.2.5.3.4 Discussion: Lactose analysis

The MilkoScan™ Minor lactose measurements did not correlate with the reference method. This was not unexpected and suggests that the oligosaccharide component of the milk is responsible for more of the individual variation than the lactose content. Previous attempts to calibrate the MilkoScan™ Minor using an enzymatic assay to measure lactose, have also been unsuccessful (158).

We therefore decided to compare the CHO reading using a different reference method, one which would account for the oligosaccharide content of the milk. The only method readily available to do this was to measure CHO by difference.

2.2.5.4 Method: CHO analysis

Ten samples of human milk were analysed at the National Measurement Institute (NMI) Melbourne (NATA accredited laboratory) for CHO by difference at, and then compared with the MilkoScan^{TM} Minor analysis. Carbohydrate was calculated from the following equation:

CHO = 100 – (fat + protein + ash + moisture) g/100 mL

The following methods were used by the NMI:

Fat:	Mojonnier method. Australian Standard 2300.1.3. (167) Official Methods of Analysis of the Association of Official Analytical Chemists International (AOAC), 16th Edition 95402,948.15,922.08
Protein:	Kjeldahl method. Australian Standard 2300.1.2.1(163) AOAC 16th Ed. 981.10, 920.152, 990.03, 920.87
Ash:	**'**Determination of Ash in Food VL 286' AOAC, 16th Edition, 1995, 923.03 and 900.02
Moisture:	'Moisture Determination in Food' Australian Standard 2300.1.1 AOAC 16th Ed. 934.06, 964.22

2.2.5.5 Results: CHO analysis

The results from two samples were discarded due to a malfunction of the MilkoScan^{TM} Minor. Results for the eight available samples are reported in **Figure 2-5**:

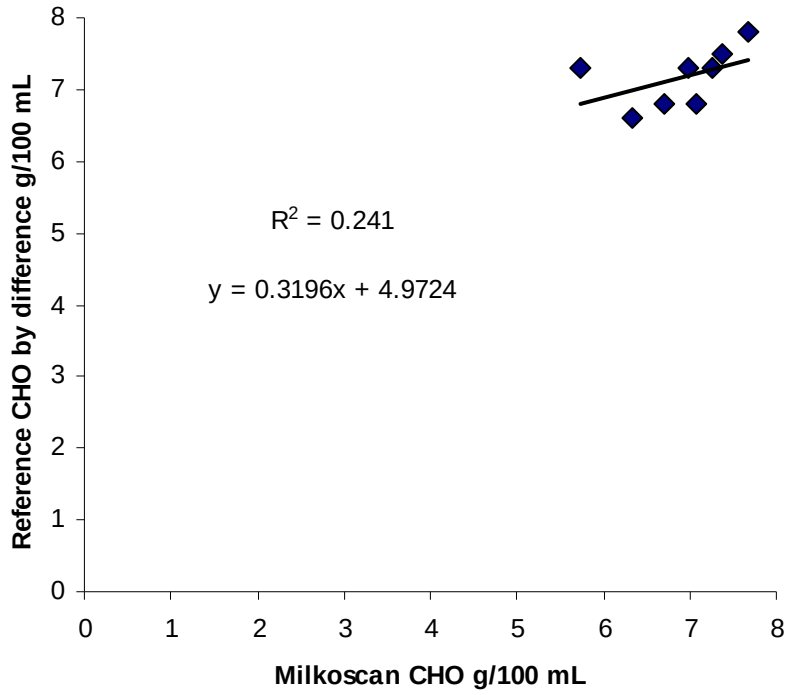


Figure 2-5 Carbohydrate: Reference (by difference) vs Measured (MilkoScan) (n = 8)

The correlation using this method is an improvement on the lactose assay but still does not allow reliable calibration.

2.2.5.6 Discussion: CHO

The MilkoScan™ Minor measurement for the carbohydrate fraction of human milk has previously been difficult to validate with a reference method (158) and our experience was similar. This is probably due to the complex mix of carbohydrates present in human milk and the limitations of available reference methods. Our initial aim was to calibrate the analyser for lactose, the nutritionally available CHO, but this was not possible given the poor correlation coefficient. The 'CHO by difference' method improved the correlation coefficient but not enough to reliably calibrate the analyser. CHO by difference is a relatively crude

way of measuring CHO because by definition it is the residual from four other analyses and so tends to compound the errors in the techniques used. For these reasons, the MilkoScan^{TM} Minor was not re-calibrated on the basis of these experiments. The lactose content of human milk remains relatively stable at approximately 6.8 g/100 mL while the oligosaccharide content varies between individuals (165). We therefore decided that, for the purposes of this study where the CHO concentration of human milk would be used only to estimate dietary macronutrient and energy intake, we would use a constant factor of 6.8 g/100 mL for the lactose content of human milk.

2.2.6 Precision

Precision was evaluated by both intra- and inter-assay variation experiments.

2.2.6.1 Methods of the intra-assay variation experiment

Intra-assay variation was assessed by running 20 replicate samples of human milk at 3 different protein concentrations within the same run (20 x 3 = 60 samples). The protein concentrations were representative of the lower, average and upper levels expected in human milk. Statistical analysis was performed using Microsoft Excel software to calculate the mean, SD and the co-efficient of variation (CV) for each protein level. The co-efficient of variation was calculated as:

CV = SD / mean x 100%

2.2.6.2 Results

Results of the intra-assay experiment are presented in **Table 2-1**.

Table 2-1 MilkoScan^{TM} Minor intra-assay means, standard deviations and coefficients of variation

	Means, Standard Deviations & Coefficients of Variation								
	Low Protein (n=20)			**Medium Protein** (n=20)			**High Protein (n=20)**		
	Mean	**SD**	**CV**	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**
	g/100mL	g/100mL	**%**	g/100mL	g/100mL	**%**	g/100mL	g/100mL	**%**
Protein	0.95	0.01	0.54	1.41	0.01	0.65	2.46	0.01	0.59
Fat	2.69	0.09	0.68	2.91	0.02	0.57	3.28	0.01	0.41

The coefficients of variation are below 1 for protein and fat, at all levels of protein studied, indicating that the precision of the instrument is high.

2.2.6.3 Methods of the inter-assay variation experiment

Inter-assay variation was established by running replicate samples, one each of 3 different protein concentrations, every day for 22 consecutive days (3 x 22 = 66 samples). The milk was thoroughly mixed on a Vortex for 60 seconds and then aliquotted under agitation to containers using a sterile burette and frozen at -20ºC until needed. They were then defrosted in the refrigerator overnight, brought to room temperature and mixed on a Vortex for 30 seconds immediately prior to analysis.

2.2.6.4 Results

Results of the inter-assay experiment are presented in **Table 2-2**. One result from the high protein group was discarded due to a malfunction of the analyser during that analysis (n=21). The CVs for protein and fat are <2% and <5%, respectively indicating good intermediate precision for protein which is the focus of this thesis.

Table 2-2 MilkoScan™ Minor inter-assay means, standard deviations and coefficients of variation

	Means, Standard Deviations & Coefficients of Variation								
	Low Protein (n=22)			**Medium Protein** (n=22)			**High Protein (n=21)**		
	Mean	**SD**	**CV**	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**
	g/100mL	g/100mL	%	g/100mL	g/100mL	%	g/100mL	g/100mL	%
Protein	0.82	0.02	1.96	1.4	0.01	1.03	2.45	0.03	1.13
Fat	2.08	0.11	5.18	4.00	0.38	9.43	4.64	0.08	1.69

2.2.6.5 Discussion of intra- and inter-assay variation experiments

The repeatability of the instrument, as assessed by intra-assay variation, is high, with CVs less than 0.7 for protein and fat across the range of concentrations tested.

The intermediate precision of the analyser, as assessed by inter-assay variation, was also high for protein with CVs less than 2%. However, the CV for fat was higher, although still <10%. Examination of these results showed two samples in the medium protein group that were significantly different to the others and these occurred just prior to the analyser malfunction. When these two samples are excluded from the analysis, the CV for fat is 1.47. It is also likely that the higher

CVs for fat reflect the rapid separation of fat in breast milk and therefore the difficulties of successfully proportioning a sample into truly representative aliquots despite the care taken.

2.2.7 Quantitation Limit

The QL for protein was calculated from the slope of the calibration curve (S) and the SD of human milk of a known protein concentration, diluted to an estimated QL. Five samples of human milk diluted to approximately 0.2 g protein/100mL were used to calculate the SD.

The following equation was used:

$$QL = \frac{10SD}{S}$$

$$\text{I.e. } QL = \frac{10 * 0.03}{1.026} = 0.3g/100ml$$

2.2.8 Quality Control: Protein standards

As this thesis is primarily about protein, the development of protein standards representative of lower, average and upper limits was important for quality control and future calibration of the MilkoScan^{TM} Minor. The fat content of these standards was also assessed. However, as the protein standards were comprised of pooled milk; little variation in the fat content of the three standards was expected.

Three standards were created at low, medium and high concentrations of protein by pooling milk into the three categories. An aliquot of each standard was analysed using the method described in **Section 2.2.5.1** by Dairy Technical

Services Ltd., Flemington, Victoria, Australia. Twenty other aliquots of 8mL of each standard were frozen at –20^{o}C for later quality control. A Levy-Jennings chart was developed for each protein standard using the reference value as the mean and using the SD from the inter-assay experiment as detailed in **Section 2.2.6**. 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. The chart monitored performance over time and drifts from the mean could be observed.

2.2.9 Challenges

Calibrating the MilkoScan^{TM} Minor presented many challenges as the machine malfunctioned numerous times during the course of the experiments. This involved several visits from the company technician to diagnose the problem and upgrade software and various components. With each service, rigorous quality control was undertaken to ensure that the changes had not interfered with our calibrations. At one stage, the machine was returned to the manufacturer in Denmark for repairs which included a new infra-red detector (a major component of the analyser) and consequently, internal recalibration of the MilkoScan^{TM} Minor against the company's reference infra-red spectrum database. On return, it was necessary to repeat the comparison of methods study to ensure validity of the human milk measurements. This resulted in a slightly different slope and intercept and the analyser was adjusted using these values.

2.3 Conclusion

Table 2-3 summarises the performance of the MilkoScan™ Minor as a technique to measure the macronutrient content of human milk.

Table 2-3 Performance summary MilkoScan™ Minor

Technology	Infrared technology with wavelengths from 1 – 12 µm	
Sample volume	8 mL	
Time for analysis	90 seconds	
Precision	**Protein**	**Fat**
Intra-assay CV	< 0.6%	< 0.7%
Inter-assay CV	< 2%	approx 5%
	Protein	**Fat**
Slope and Intercept prior to repair	y = 1.03 – 0.3 (R^2 = 0.98)	y = 1.03 + 0.3 (R^2 = 0.98)
Slope and Intercept post repair	y = 1.06 – 0.6 (R^2 = 0.98)	y = 1.03 + 0.3 (R^2 = 0.98)
Quantitation Limit	Protein 0.3 g/100 mL	
Range	**Protein**	**Fat**
	0.3 – 5 g/100 mL	0.78 – 8.8 g/100 mL

The MilkoScan™ Minor was successfully calibrated to measure the protein and fat content of human milk. CHO validation was not successful. However, for nutritional studies, using a constant of 6.8 g lactose /100mL is adequate to estimate total energy intake (158). This technique was used to analyse human milk samples used in the clinical trial undertaken for this thesis.

Chapter 3 Methods of a double blind randomised controlled trial of protein fortification of human milk

3.1 Trial design

3.1.1 Introduction

This chapter describes the design and methods of the randomised controlled trial (RCT) of protein fortification of EBM in preterm infants born <31 weeks gestation. The trial compares growth of infants receiving HMF providing 1.4 g protein /100 mL EBM (the higher protein group) with HMF providing 1.0 g protein/100 mL EBM, which is equivalent to current clinical practice. This pragmatic trial was designed to test the efficacy of the intervention in existing nursery routines and acknowledges that preterm infants fed EBM may also receive a varying amount of preterm formula. The start of the study period was defined as the first day the infant received the intervention and study end was at discharge or EDD, whichever came first. Approval to conduct the study was granted from the Children, Youth and Women's Health Human Research Ethics Committee at the WCH, the Flinders Clinical Research Ethics Committee at FMC and the Ethics of Human Research Committee, Central Northern Adelaide Health Service for Lyell McEwen Health Service and Modbury Hospital (two major transfer hospitals). Approval for the trial from the other down transfer hospitals was granted by the medical and nursing Heads of Department and these hospitals were:

- Port Augusta Hospital and Regional Health Service
- Mt Gambier and Districts Health Service

- Ashford Hospital
- Riverland Regional Hospital

3.1.2 Objectives and Hypotheses

The objective of this study was to increase the protein concentration of fortified human milk to levels thought to meet requirements (at least equivalent to current preterm formula), while maintaining the same energy level, and assess the effect on growth.

3.1.2.1 Primary hypothesis

Preterm infants fed EBM fortified with a higher level of protein (1.4 g/100 mL) will have improved length gain (cm/week) when compared with infants fed EBM fortified at standard levels (1.0 g/100 mL).

3.1.2.2 Secondary hypotheses

Preterm infants fed EBM fortified with a higher level of protein (1.4 g/100 mL) will have:

- Improved weight and occipital head circumference (OHC) gains
- higher biochemical markers of protein metabolism (BUN, albumin)
- but no increase in biochemical disturbances or adverse events

when compared with infants fed EBM fortified at standard levels (1.0 g/100 mL).

3.1.3 Outcomes

3.1.3.1 Primary outcome

The primary outcome for this trial was length gain. Length was selected over other growth parameters as, unlike weight gain, it is not affected by hydration status (168) and fat tissue accretion.

3.1.3.2 Secondary outcomes

Secondary growth outcomes were weight and OHC gains for the duration of the study (HMF introduction to discharge home or EDD, whichever came first).

Secondary safety outcomes included biochemical markers of protein and amino acid metabolism, and feeding tolerance and clinical morbidity. These are described in more detail later in this chapter. The biochemical data included plasma levels of urea, creatinine and albumin, and blood pH and amino acid levels. Clinical data included the incidence of NEC, sepsis and retinopathy of prematurity (ROP), indicators of respiratory function, development of brain injury, surgery and length of hospital stay. Feeding and tolerance data included the time spent on parenteral nutrition and intravenous lipid, time taken to reach full enteral feeds and the volume of enteral intake when HMF was introduced.

3.1.4 Participants

3.1.4.1 Setting and location

The WCH, North Adelaide, South Australia was the primary site for the trial. Due to lower than anticipated recruitment rates, the trial was extended to FMC, Bedford Park, South Australia. These two hospitals provide the only tertiary

neonatal care in South Australia and provide a neonatal retrieval service to a large geographical area covering South Australia and parts of the Northern Territory. WCH Neonatal Unit is comprised of a 14 bed Neonatal Intensive Care Unit (NICU) (Level III) and a 35 bed Special Care Baby Unit (SCBU) (Level II) staffed by 5 Consultant Neonatalogists, Registrars and 3 Neonatal Nurse Practitioners. The FMC Neonatal Unit contains 11 intensive care cots and 24 Special Care cots staffed by 4 Consultant Neonatalogists, Registrars and 2 Neonatal Nurse Practitioners. It is usual for some infants to be transferred for ongoing care to Level II or I nurseries at peripheral hospitals closer to their home. Six peripheral hospitals located in either the Adelaide metropolitan area, or close rural areas participated in this trial.

3.1.4.2 Participants

The target population of preterm infants born <31 weeks gestation was chosen because the smallest, least mature infants use fortifiers for the longest time. They are also more vulnerable and possibly more responsive to intervention. We estimated the length of stay, from a previous study in our unit, as an average of six weeks in this population (33). Exclusive breastfeeding is impossible to predict in advance and not representative of the neonatal population. This trial was designed to reflect current clinical practice, so mothers were approached if they intended to breastfeed regardless of whether or not they were expressing the full volume of EBM required at enrolment to meet their infant's needs.

3.1.4.2.1 Inclusion criteria

All infants <31 weeks gestation who were born at, or retrieved to, the NICU at either WCH or FMC and whose mothers intended to supply breast milk (either exclusively or partially), were eligible for inclusion in the trial. Enrolment was within 3 days of receiving any fortified feeds.

3.1.4.2.2 Exclusion criteria

Infants were not considered for the trial if any of the following criteria were present:

1. major congenital or chromosomal condition known to affect growth
2. any condition where extra protein is contraindicated
3. a low maternal supply of breast milk together with an uncertainty about continuing to express milk
4. residence in a remote rural area with a Level II nursery where follow-up would be difficult

3.1.5 Randomisation

3.1.5.1 Sequence generation

The randomisation schedule was stratified for sex and GA (<28 weeks and 28 to 30 weeks) as the primary outcome (length gain) is known to be influenced by both of these variables.

Two independent staff members at CNRC, not involved in the trial, developed the randomisation schedule prior to the commencement of the trial, using a computer random number generator to select random permuted blocks of four. This schedule and the block lengths were unknown to any research staff involved in the

trial. Multiple births were randomised to the same group according to the sex of the first born infant.

3.1.5.2 Allocation concealment and implementation

Prior to trial commencement, the tins of fortifier (which were identically packaged) were sequentially numbered according to the allocation sequence by the 2 independent staff members who had generated the randomisation schedule. The number on the tin became the infant's study identification number. The tins were placed in four boxes according to their strata:

- Females <28 weeks gestation
- Females 28 to 30 weeks gestation
- Males <28 weeks gestation
- Males 28 to 30 weeks gestation

Once consent was obtained, the next sequentially numbered tin of fortifier from the appropriate box was taken, labelled with the infant's name and delivered to the milk preparation room where the fortified milk was made. The group allocation was not revealed until after recruitment, data collection and statistical analyses of the primary outcome were completed.

3.1.5.3 Implementation

Eligible infants were identified by the research midwives in the unit. Parents were then approached initially by a neonatologist who explained the trial and provided an information sheet. If, after consideration, the parents decided to participate in the trial, they were enrolled by the author or one of the research midwives and asked to sign a consent form. The intervention was then allocated as described

above, usually by the author, sometimes by the research midwives and, on rare occasions, by other members of the CNRC, but not those responsible for generating the randomisation schedule. For quality assurance, all allocations were ratified by a second person.

3.1.6 Blinding

All study personnel, clinical staff including those in the milk preparation room, and participants involved in the trial were blinded to the intervention for the duration of the study. The study fortifiers were identical in colour, form, rate of mixing and packaging. Preliminary statistical analyses of growth were performed blinded: an independent staff member coded participants into 'Group A' or 'Group B' but the control and intervention groups were not identified.

3.1.7 Interventions

Both fortifiers were made and donated by the Nestlé Product Technology Centre in Konolfingen, Switzerland and were based on their commercially available fortifier, FM85. The fortifiers consisted of hydrolysed whey protein, maltodextrin, vitamins and minerals. CHO levels were adjusted to make the fortifiers isocaloric. Energy and fat content of the fortifiers were the same. The fortifiers were identically supplied as a powder in 100 g tins, and both were mixed with EBM at a rate of 1 g/20 mL. Composition of the study products is given in **Table 3-1**. The addition of extra protein decreased the solubility of the mineral mix so that it was not technically possible to achieve an identical mineral composition between the two fortifiers. However, all

minerals were supplied at adequate levels and so these differences are unlikely to affect growth.

The interventions were HMF containing either 1.4 g protein/100 mL of EBM or 1 g/100 mL. After randomisation, the appropriate tin of fortifier, labelled with the infant's name, was stored in the milk preparation room. The nurse dedicated to the daily milk preparation matched the EBM (fresh, or frozen and defrosted) to the appropriate tin of fortifier and prepared the fortified milk. Only mother's own milk was used. If the supply of EBM was insufficient, preterm infant formula was used. Formulae used at WCH were Karicare Nutriprem RTF (Nutricia), prior to March 2007 and S26 LBW (Wyeth) after March 2007. FMC used PreNan (Nestle). All of these preterm formulae contained 80 Kcal/100mL and 2.2 to 2.4 g protein/100mL. Special formulae were used rarely and only when clinically indicated and included Neocate (SHS) (2 g protein and 71 Kcal/100mL) Monogen (SHS) (2 g protein and 74 Kcal/100mL) and Karicare Delact (Nutricia) (1.7 g protein and 70 Kcal/100mL).

Table 3-1. Composition of Trial Fortifiers

Component /100g powder		HMF 1.4g protein/100mL 'Higher Protein'	HMF 1g protein/100mL 'Standard protein'	% Difference if > 10%
Energy	Kcal	343	343	
	kjoules	1435	1435	
Solids	g	96	96.2	
Fat	g	0.6	0.6	
Protein (equivalent)	g	27.5	19.8	
Carbohydrates	g	57.2	64.9	
Maltodextrins	g	56	64.5	
Ash	g	6.8	6.5	
Na	mg	426	406	
K	mg	1080	800	**+26**
Cl	mg	260	320	**-19**
Ca	mg	1344	1447	
P	mg	840	891	
Mg	mg	39	49	**-20**
Mn	µg	108	130	**-17**
Se	µg	36	31	**+17**
Ratio Ca / P		1.6	1.6	
Vit A	IU	12313	11736	
Vit D	IU	2191	2152	
Vit E	IU	60	68	**-11**
Vit K	µg	90	83	
Vit C	mg	217	196	
Vit B1	mg	1.1	1.1	
Vit B2	mg	2.2	2.2	
Niacin	mg	17.9	17.6	
Vit B6	mg	1.2	1.2	
Folic acid	µg	853	880	
Vit B5	mg	8.5	8.8	
Vit B12	µg	2.1	2.4	
Biotin	µg	65.2	63.6	
Fe	mg	20	29	**-31**
I	µg	335	323	
Cu	mg	0.6	0.9	**-33**
Zn	mg	11.7	16.6	**-30**
Cr	µg	12.7	17.6	**-28**
Mo	µg	5.3	7.3	**-27**
F	µg	68	65	

3.1.8 Feeding guidelines

Feeding guidelines were developed and documented for this study to ensure consistency between clinicians and sites. Goals were to achieve full enteral feeds, defined as ≥150 mL/kg/day, by the second week of life, to regain birth weight by day 10 to 14, and to achieve a weight gain of 14 to 15 g/kg/day. Trophic feeds were commenced as soon as cardiopulmonary status was stable (day 1 or 2 where possible) at 5 – 10 mL/kg/day using EBM or preterm formula if EBM was unavailable. Bolus gravity feeds were initiated and tolerance was assessed on an individual basis, with a change to continuous feeds if necessary. HMF was added to EBM when the enteral intake reached ≥80 mL/kg/day or when tolerance was demonstrated. Feeds were advanced at a rate of 10 to 20 mL/kg/day if tolerated, to a final rate of 160 to 180 mL/kg/day. Cessation of HMF was not defined. However, the usual practice for this trial was to continue using HMF until study end, defined as discharge or EDD, whichever came first, for whatever proportion of the milk was given as EBM via a bottle or enteral tube. In practice, most infants progressed to sucking feeds; either from the breast, formula from a bottle, or a mixture of both, by the end of the study.

3.1.9 Collection of trial data

Data were collected from the parents and the neonatal case notes to determine baseline demographic and clinical characteristics.

Dietary intake data and weight, from detailed fluid balance charts used clinically, were collected daily by the author and the research midwives. Details recorded

included the volume, caloric density, and supplements added, for both breast milk and formula. Macronutrient intakes were calculated from the volume of milk ingested, the protein concentration of EBM and the manufacturers' information regarding the study fortifiers and any formula consumed. The milk room prepared a 24-hour volume of fortified EBM daily from supplies provided fresh by the mother and supplemented with frozen own mother's milk as necessary. A weekly sample of this EBM, before fortification, was analysed using the MilkoScan™ Minor as described in **Chapter** 2 and was used as the representative composition for the week. The energy content of EBM was calculated using the Atwater factors of 17 (4), 17 (4), and 37 (9) kjoules (Kcal)/g for protein, lactose and fat, respectively.

The WCH neonatal unit provide a Neonatal Early Discharge (NED) programme where parents of infants nearing discharge who are clinically stable, but still establishing feeding, may elect to go home with support from a team of visiting midwives. This is classified as 'hospital in the home' and so is included as part of the hospital admission. Midwife visits were initially daily, then tapered to twice weekly until discharge. Parents on my trial who elected for the NED programme were trained, and provided with the appropriate equipment, to prepare the fortified milk and to document the daily dietary intake. Anthropometric measures continued on the NED programme: weight was measured twice weekly by the visiting midwife using portable electronic scales and the author visited with the midwife weekly to measure length, using a recumbent length board, and OHC.

Some infants were transferred to other hospitals providing level II care in the metropolitan or close rural area where the trial continued. The author provided support for each of these hospitals for the trial by in-service training to both the medical and nursing staff, a folder of reference trial information, equipment needed for the milk preparation and weekly visits. At these visits, the author delivered stocks of the fortifier, reviewed the medical records, sampled the EBM and collected feeding, anthropometric and clinical data.

For quality assurance, at trial completion data were reviewed and ratified by senior staff at the WCH neonatal unit: a neonatologist, a neonatal nurse practitioner, or a senior neonatal researcher. In particular, the clinical data (**Section 3.3**) were reviewed for accuracy of the diagnoses.

3.1.10 Sample size

A study using adjusted fortification versus standard fortification with a difference in protein of approximately 0.4 g/100mL showed a non significant increase in length gain of 0.14 cm/week with a standard deviation 0.28 cm (127). Using repeated measures, we estimated that a sample size of 80 would detect a difference of 0.13 cm/week with a power of 88% and a two-sided significance of 5%.

3.1.11 Statistical methods

Analyses were done on data from all babies randomised according the allocated group and probability <0.05 was considered significant.

The statistical analyses for anthropometric and biochemical data, the volume of intake and protein intake from milk fortifier were outsourced to the Data Management and Analysis Centre (DMAC), The University of Adelaide and performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Differences between the groups for length, weight and OHC gains were assessed with a repeated measures model using Generalised Estimating Equations (GEEs) to adjust the variance for clustering on siblings (multiple births) and measures within infants over time. The model included the fixed effects of intercept, study day, treatment group and the interaction between study day (SD) and treatment group. The model also adjusted for the covariates sex and GA. Age prior to intervention was not used as an adjusting variable because it can be seen as a post-randomisation variable. Differences in growth between twins and non-twins was explored and found not to be significant. The model form was as follows:

*Outcome = intercept + SD + Treatment + GA + Sex + SD*Treatment + Error*

with clustering on siblings and repeated measures on infants.

Diagnostic plots of the residuals were used to assess the fit of the model.

Analysis of a subgroup of infants who complied with the protocol was planned *a priori*. Compliance was defined as those infants receiving ≥70% of their measured enteral intake as fortified breast milk. We did not measure the volume of milk ingested from direct breast feeds, so 'measured enteral intake' refers to the volume given via an enteral tube or by sucking from a bottle. This analysis was restricted to the primary outcome of length gain.

Secondary *post hoc* exploratory analyses were performed for the primary outcome only and limited to the stratification i.e. sex and GA (<28 weeks, 28 – 30 weeks).

The statistical analyses for the nutritional management and clinical outcome data were done by me using either SPSS for Windows (Version 15,0,0, Chicago Il, USA) prior to November 2009 and PASW (Version 18.0.0, Chicago Il, USA) after November 2009. Continuous normally distributed variables were compared using independent samples T tests.

3.2 Anthropometric measurements

All anthropometric measurements were taken by two people with each observer independently reading and recording the measurement. One observer positioned the infant while the other took the measurements, and then the roles were reversed. The two observers usually came from a trained team at each site, consisting of me and experienced neonatal research midwives (two at WCH and one at FMC). On occasions where two trained assessors were not available, one trained assessor was used in conjunction with the infant's primary nurse. All assessors received training according to the World Health Organisation (WHO) Child Growth Standards Training Course (169). Inter-observer variability was measured during the trial as the deviation from the author's measurements, because the author was the common observer between sites. These inter-observer variability results are reported in **Chapter** 4**.** A protocol modified from the WHO Multicentre Growth Reference Study (170) was developed for all anthropometric measures and is described below.

3.2.1 Length

Length was measured weekly in duplicate to the nearest 1 mm using a recumbent length board (O'Leary, Ellard Instruments, CA). The nappy was removed to ensure the infant's legs could be held together and straightened. One assessor supported the infant's head in the Frankfort plane and applied gentle traction to bring the top of the head in contact with the fixed headpiece. The infant's hips and shoulders were positioned at right angles to the long axis of the body. Simultaneously, the other assessor aligned the infant's legs by placing one hand gently but firmly over the knees. Toes were positioned pointing directly upward with the soles of the feet perpendicular to the horizontal footboard of the measuring device. Gentle pressure was applied at the knees to straighten the legs. The recorder then slid the moveable foot piece to rest firmly at the infant's heels and length was recorded to the nearest 1 mm. The two assessors then traded positions and the process was repeated. Both measurements were recorded and the average was used as the final value. Measurements were repeated if there was a discrepancy of more than 7 mm.

3.2.2 Weight

Infants were weighed using electronic balance scales accurate to 5 g. The scales were calibrated annually using standard weights. One measurement of naked body weight was taken at approximately the same time each day when the infant was in NICU and then twice weekly in SCBU.

3.2.3 Occipital head circumference

OHC was measured using a single use paper tape. The tape was placed across the frontal bones just above the eyebrows, around the head above the ears on each side, and over the occipital prominence at the back of the head. The tape was moved up and down over the back of the head to locate the maximal circumference of the head. The tape was placed perpendicular to the long axis of the face and pulled gently to compress the hair and underlying soft tissues. Two measurements were taken weekly by independent assessors and recorded to the nearest 1 mm. Measurement differences >5 mm were repeated and the final value used was the average of the two measurements.

3.3 Clinical data

Clinical definitions were generally consistent with the Australian and New Zealand Neonatal Network (171) and are defined below.

3.3.1 Necrotising enterocolitis

Proven NEC was defined as having at least four of the following symptoms including at least one systemic and one intestinal sign: instability, apnoea, bradycardia, lethargy, a residual of more than 25% of the previous feed on two consecutive occasions, abdominal distension, vomiting or faecal blood. The infant was required to have had a profile consistent with NEC including at least one of the following: abdominal wall cellulitis and palpable abdominal mass, or pneumatosis intestinalis, or portal vein gas, or a persistent dilated loop on serial x-

rays, or a surgical or post mortem diagnosis. The infant needed to have warranted treatment for NEC, which included nil by mouth and antibiotics.

3.3.2 Sepsis

Sepsis was defined as isolation of an organism from at least one blood culture that required antibiotics. Mixed coagulase negative staphylococci or other skin flora-contaminants are not included. Antibiotic use without a positive blood culture was not counted as sepsis.

3.3.3 Respiratory function

The respiratory data collected included the number of days each on endotracheal support, continuous positive airway pressure (CPAP) support and requiring oxygen. Chronic lung disease was assessed by oxygen requirement both at 36 weeks PMA and discharge home or at EDD whichever came first.

3.3.4 Retinopathy of prematurity

Routine screening for ROP was done by a paediatric ophthalmologist and the most severe stage in each eye documented. The classifications used were:

Stage I demarcation line

Stage II ridge

Stage III ridge with extraretinal fibrovascular proliferation

Stage IV retinal detachment

The use of cryotherapy or laser treatment was also recorded.

3.3.5 Brain injury

Intraventricular haemorrhage (IVH) seen on cranial ultrasound was graded as follows:

Grade I: subependymal germinal matrix IVH

Grade II: IVH with no ventricular distension

Grade III: the ventricle is distended with blood

Grade IV: intraparenchymal haemorrhage

Periventricular leukomalacia (PVL) injuries were also identified by cranial ultrasound and characterised by brain hemisphere and region (occipital, parietal and frontal).

3.3.6 Tolerance

The number of days on which feeds were interrupted was recorded with partial days counted as one. Other information recorded included the number of days to reach full enteral feeds, defined as 150 mL/kg, days of parenteral nutrition and intravenous lipid. The days of age and volume of enteral intake when HMF was introduced were also recorded.

3.3.7 Other clinical data

Any surgery the infant underwent was recorded as was the use of postnatal steroids. Length of stay was documented until EDD if infant was still an inpatient and included time spent at peripheral hospitals or on the NED programme where relevant. The number of days spent in level III care was also recorded, counting

part days as one and days on which transfer occurred counted as the highest level of care.

3.4 Biochemical data

For the duration of the study, blood samples (500 µL) were taken by heel prick, weekly initially and then every 2 weeks when the infants were in level II care. Clinical staff had access to these results as part of their routine clinical care. The samples were analysed at their respective hospital laboratories with the exception of whole blood amino acids which were all analysed at the Neonatal Screening Laboratory at WCH. The following metabolites were analysed and are discussed in more detail below:

- SUN (mmol/L)
- plasma creatinine (µmol/L)
- plasma albumin (g/L)
- blood pH

3.4.1 Serum urea nitrogen

SUN was used in this trial both as an indicator of protein nutritional status and also as a marker of excess protein (153-155). SUN was determined spectro-photometrically using a urease method produced by Roche. The same analysers were used for urea, creatinine and albumin determination. The WCH used a Roche Cobas 6000 Analyser and FMC used a Roche/Hitachi Modular Analyser, (Roche Diagnostics GmbH, Mannheim, Germany). Both laboratories are

accredited by the National Association of Testing Authorities (NATA), Australia. The precision (CV) of the assay was <2.2% at FMC and 1.8% at WCH.

3.4.2 Creatinine

Plasma creatinine levels were used as a marker of renal function in this trial. Plasma creatinine levels were determined spectro-photometrically using an enzymatic colorimetric method produced by Roche on the same equipment as above. The manufacturer compared values determined on a Roche/Hitachi Cobas c 501 Analyzer with those determined using the same reagent on a Roche/Hitachi 917 Analyzer and the linear regression of the two methods was:

$y = 0.996x + 1.18$ μmol/L and $r = 1.000$

The CV for the assay was <2% at FMC and <3% at WCH.

3.4.3 Plasma albumin

Plasma albumin levels were used in this trial as one indicator of protein nutritional status. It has a long half life of approximately 20 days and so represents long term changes in nutritional status. Plasma albumin levels were determined colorimetrically using a Bromocresol Purple method produced by Roche on the same equipment as above. The manufacturer compared values determined on the Roche/Hitachi Cobas c 501 Analyzer with those determined using the same reagent on a Roche/Hitachi 917 Analyzer and the linear regression of the two methods was:

$y = 1.021x + 0.01$ g/L and $r = 0.997$.

The CV of the assay was 3% at WCH and <1.5% at FMC.

3.4.4 Blood pH

Blood pH was used as a measure of respiratory or metabolic acidosis. Levels were determined in each neonatal unit, using a blood gas analyser. WCH used an ABL700 Series (Radiometer Medical ApS, Brønshøj, Denmark) and FMC used an ABL800 Flex analyser (Radiometer Medical ApS, Brønshøj, Denmark).

3.4.5 Whole blood amino acids

Whole blood amino acids were measured in this trial to determine the incidence of hyperaminoacidaemias and to determine differences between the groups. The potentially neurotoxic amino acids phenylalanine and tyrosine were measured. The branched chain amino acids (leucine, isoleucine and valine) were also measured. As leucine and isoleucine have the same atomic weight, it is not possible to separate out these two amino acids using tandem mass spectrometry. For ease of comparison, the sum of the branched chain amino acids (leucine, isoleucine and valine) were compared between groups.

A spot of whole blood was collected on filter paper (S & S 903 filter paper; Schleicher & Schüll, Dassel, Germany) and all samples were analysed for amino acids at the Neonatal Screening Laboratory (WCH) using Tandem Mass Spectrometry (Micromass Quattro II tandem mass spectrometer). Tandem Mass Spectrometry quantifies blood metabolites according to their mass-to-charge ratio.

Chapter 4 Efficiency of higher protein fortifier on preterm infant growth

4.1 Introduction

This chapter describes the trial participants, dietary intake, feeding practices and anthropometric results from the RCT whose methods are detailed in **Chapter Chapter 3**. The Consolidated Standards of Reporting Trials (CONSORT) statement (172, 173), and the extension relating to pragmatic trials (174), have been used as a framework for the structure of this chapter. These statements are designed to improve reporting of RCTs and to facilitate consideration of bias and external validity. Participant flow through the study and baseline characteristics of the participants are described, followed by the dietary intake and feeding practices. The anthropometric outcomes for the study are then presented.

4.2 Participant flow

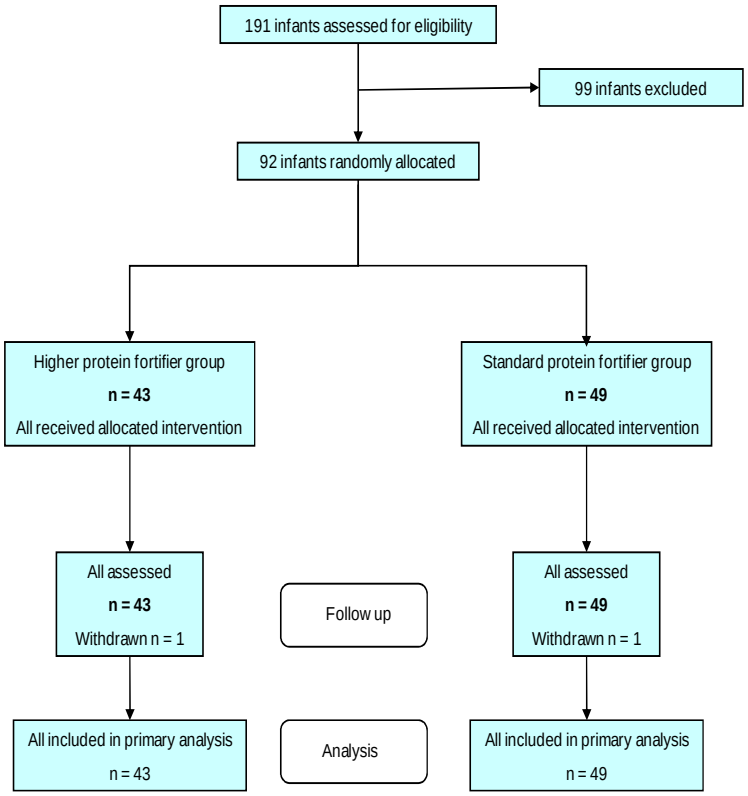
One hundred and ninety one infants were evaluated for potential enrolment. Fifty three infants were ineligible and 21 infants were not approached. Of the 117 eligible infants, parents of 25 declined to participate (**Table 4-1**). The most common reasons given for declining to participate in the study were that the parents did not want to be involved in research in general or wanted to avoid extra blood tests. Parents of 92 infants agreed to participate and were randomised as shown in Figure 4-1.

Table 4-1 Reasons given for exclusion from trial

Reason	**n = 99**
Ineligible, n, (%)	53 (54)
Inadequate milk supply	30
Outside Adelaide metro area	17
Congenital defects	4
Fortified feeds for >3 days	2
Not approached, n, (%)	21 (21)
Missed for logistical reasons	13 (13)
Mother or infant too ill or distressed to	5 (5)
approach	3 (3)
Other reasons	
Declined to participate, n, (%)	25 (25)

Eighty infants were recruited from the WCH and twelve from FMC. Forty nine infants were randomised to the control group and forty three to the intervention group. All infants initially received their allocated intervention. Two infants were withdrawn at the parents' request due to gastrointestinal intolerance (one from each group) and these infants were given the standard ward fortifier (S-26.SMA HMF, Wyeth Nutritionals, Georgia, Vermont). However, the parents consented to the ongoing collection of growth measurements and so all infants were included in the primary outcome analysis.

Figure 4-1 Participant flow through the study



Recruitment at the WCH was undertaken from October 2006 to March 2008. Due to a lower than average number of preterm births during this time, the recruitment rate fell below our target, and consequently the trial was extended to FMC from July 2007 to March 2008. The last participant completed the trial in June 2008.

4.3 Demographic and clinical characteristics at randomisation

4.3.1 Maternal pregnancy and birth characteristics

Maternal characteristics are shown in **Table 4-2**. Maternal age, parity and previous preterm birth experiences were comparable between the groups. In-vitro

fertilisation (IVF) conception was more common in the standard protein group and this group had a higher incidence of twins (see **Table 4-4).** Of the 14 infants conceived by IVF in the standard protein group, 12 were twins and 2 were singletons.

Table 4-2 Maternal age, pregnancy and birth characteristics of study participants

	Higher protein (n = 43)	**Standard protein (n = 49)**
Mean maternal age, ± SD, *years*	29 ± 6	30 ± 5
Parity, *n (%)*		
Primiparous	20 (49)	19 (49)
Multiparous, (*range)*	21 (51) (2–5)	20 (51) (2–4)
Previous preterm birth, *n (%)*	6 (15)	5 (13)
Primary reason for preterm birth, *n (%)*		
Rupture of membranes	7 (17)	6 (15)
Preterm labour	15 (37)	14 (36)
Hypertension	7 (17)	7 (18)
Antepartum haemorrhage	6 (15)	6 (15)
Suspected IUGR[a]	3 (7)	4 (10)
Fetal distress	1 (2)	0
Other	2 (5)	2 (5)
IVF conception, *n of infants (%)*	4 (9)	14 (29)
Antenatal steroids, *n (%)*	36 (84) *(n = 42)*	45 (92)
Caesarean section, *n (%)*	27 (63)	32 (65)

[a]IUGR = intra-uterine growth retardation

4.3.2 Demographic characteristics of the parents at randomisation

The parental characteristics were similar between the two groups (**Table 4-3**). Because of twins, there were 39 and 41 parents in the standard and higher protein groups, respectively. Most participants were Caucasian (>85%). Mothers in the standard protein group had a higher level of post-school education and this was reflected in their socio-economic circumstances, with more professional women in

this group. However paternal socio-economic status (SES) was similar between the groups. More mothers drank alcohol in the standard protein group.

Table 4-3 Demographic characteristics of parents in the study

	Higher protein	**Standard protein**
Number of parents	41	39
Maternal race, *n (%)*		
Caucasian	35 (85)	37 (95)
Aboriginal	2 (5)	0
Asian	3 (7)	2 (5)
Other	1 (2)	0
Mother's highest level achieved at school, *n (%)*		
≤Year 11	9 (22)	13 (33)
Year 12	31 (76)	26 (67)
unknown	1(2)	0
Mother's highest level of education, *n (%)*		
No further study	9 (22)	10 (26)
Certificate or diploma	19 (46)	14 (36)
Degree	7 (17)	11 (28)
Higher degree	2 (5)	3 (8)
Unknown	4 (10)	1 (3)
Maternal SES		
Professional (ASCO* code 1, 2& 3)	16 (39)	22 (56)
Skilled & unskilled workers (ASCO code 4-9)	13 (32)	10 (26)
Not in workforce (unemployed, housewife,	11 (27)	7 (18)
student)	1 (2)	0
Missing		
Maternal current smoker, *n (%)*	9 (22)	5 (13)
Smoked during pregnancy, *n (%)*	9 (22)	6 (15)
Alcohol during pregnancy, *n (%)*	6 (15)	10 (26)
Up to 2 drinks/week	5	10
7-10 drinks/week	1	0
Father's highest level achieved at school, *n (%)*		
≤ Year 11		
Year 12	10 (24)	17 (44)
Unknown or missing	27 (66)	20 (51)
	4(10)	2 (5)
Paternal SES		
Professional (ASCO code 1, 2& 3)	17 (42)	17 (44)
Skilled & unskilled workers (ASCO code 4-9)	21 (51)	17 (44)
Not in workforce (unemployed, housewife,	1 (2)	2 (5)
student)	2 (5)	3 (8)
Missing		

*Australian Standard Classification of Occupations (175)

4.3.3 Neonatal characteristics at randomisation

The neonatal characteristics of the trial participants at randomisation are shown in **Table 4-4**. Less than half the infants were boys in both groups. The mean gestational ages of the groups were similar. The higher protein group included one infant born at 23 and five born at 24 weeks gestation, whereas the least mature infant in the standard protein group was 25 weeks gestation.

There were a disproportionate number of multiple births in the groups with a total of 12 multiple births enrolled in the study; 10 of which were randomised to the standard protein group and only 2 to the higher protein group. All multiple births were twins except for one in the standard protein group which was a triplet pregnancy that resulted in 2 live births. These infants were subsequently treated as twins.

Birth anthropometry and the proportion of infants classified SGA, defined as $<10^{th}$ percentile when compared with Australian reference data (176, 177), were similar between the groups. Other birth characteristics were similar between groups as were the mean number of days between birth and enrolment and between enrolment and the start of the intervention.

Table 4-4 Neonatal characteristics of trial participants

Characteristic	**Higher protein (n = 43)**	**Standard protein (n = 49)**
Recruitment hospital, *n (%)*		
WCH	37 (86)	43 (88)
FMC	6 (14)	6 (12)
Male, *n (%)*	19 (44)	21 (43)
Mean gestational age, ± SD, *weeks*	27.5 ± 2.2	28 ± 1.5
Infants born < 28 weeks, *n (%)*	23 (53)	20 (41)
Multiplicity, *n (%)*	4 (9)	20 (41)
Mean birth anthropometry ± SD		
weight, *g*	1012 ± 315	1056 ± 289
length, *cm*	35.7 ± 3.5	35.6 ± 3.4 *(n = 48)*
head circumference, *cm*	25.3 ± 2.5 *(n = 42)*	25.6 ± 2
Small for gestational age at birth, *n (%)* *		
Weight	7 (16)	6 (12)
Length	7 (16)	7 (14) *(n = 48)*
Head circumference	3 (7)	5 (10)
Mean Apgar scores ± SD		
at 1 minute	5.7 (2)	6.5 (1.9)
at 5 minutes	8 (1.6)	8.1 (1)
Resuscitation at birth required, *n (%)*	41 (95)	48 (98)
Mean days between birth and randomisation, ± SD, *days*	10.3 (5.7)	10.3 (4)
Mean days between randomisation & study start, ± SD, *days*	2.7 (3.3)	2.8 (4)

* Based on Australian birth weight data for singletons and twins by gestational age (176, 177)

4.4 Dietary intake and mode of feeding data

4.4.1 Nutritional management of infants in the trial

A feeding protocol was developed for the trial to ensure both intra-site (between clinicians) and inter-site consistency (see **Section 3.1.8**). The protocol aimed to commence trophic enteral feeds as soon as cardiopulmonary status was stable (day 1-2 if possible) and this was achieved in both groups on a median of day 3 (interquartile range 2-4). HMF was to be introduced when the enteral volume reached 80 mL/kg/day or tolerance was achieved, whereas in practice this occurred somewhat later at a median of 120 mL/kg/day for both groups

(interquartile range 96 - 156 and 94 - 140 for the higher and standard protein groups, respectively). Introduction of HMF varied between sites, with WCH introducing it at a mean (SD) intake of 122 (36) mL/kg/day and FMC introducing it at 102 (39) mL/kg/day. Another goal of our feeding guidelines was to achieve full feeds, defined as 150 mL/kg/day, by the second week of life whereas the median day (interquartile range) this was achieved was 17 (10 - 25) and 15 (12 - 18) days for the higher and standard protein groups, respectively. Overall, the nutritional management of the infants was not different between groups (**Table 4-5**).

Table 4-5 Nutritional management of study infants

	Standard protein group (n = 49)	**Higher protein group (n = 43)**
Age feeds commenced, *days*	3 (2–4)	3 (2-4)
Age HMF introduced, *days*	13 (10-16)	13 (10-18)
Volume of enteral when HMF introduced, *mL/kg/day*	120 (94-140)	120 (96-156)
Time to reach full enteral feeds*, *days*	15 (12-18)	17 (11-23)
Days on parenteral nutrition, *days*	16 (12-29)	17 (10-25)
Days on intravenous lipid, *days*	12 (8-19)	11 (7-16)

Values are median (interquartile range); * Defined as 150 mL/kg/day.
No significant differences between groups in any variables using Independent t-test

4.4.2 Intake of trial human milk fortifiers

Dietary intake data were recorded and calculated as described in **Section 3.1.9**. We made no attempt to measure the volume of human milk delivered from direct breast feeds as this may have interfered with the establishment of breastfeeding.

However, as HMF is only added to EBM, we had complete data available for the amount of HMF given and the total volume of intake from enteral tube or bottle. From this, we could calculate the protein provided by the HMF. Differences between the treatment groups over time were assessed using the model outlined in **Section 3.1.11**. **Table 4-6** shows a mean (SD) daily protein intake from human milk fortifier of 2.6 (1.2) g/day in the higher protein group and 1.8 (0.8) g/day in the standard protein group and this difference was statistically significant (p <0.0001). The mean (SD) daily volume of intake was 258 (47) mL/day in the higher protein group and 253 (53) mL/day in the standard protein group. Although this was a statistically significant difference (p = 0.03), this finding is not thought to be clinically relevant. The mean (SD) number of days in the study was 65 (17) days for the higher protein group and 61 (14) days in the standard protein group. An additional 5mL/day over 65 days accumulates to 325mL, equivalent to approximately one day's intake over the entire hospital stay, or approximately 1.5% of the total intake.

Table 4-6. Protein intake from HMF

	Higher protein (n = 43)	**Standard protein (n = 49)**	***P* value**
Mean volume intake [§] (SD), *mL/day*	258 (47)	253 (53)	p = 0.03
Mean protein from HMF (SD), *g/day*	2.6 (1.2)	1.8 (0.8)	p <0.0001

[§]Defined as intake via an enteral tube or bottle. Does not include direct breastfeeds

4.4.3 Enteral dietary intake

This trial recruited participants whose mothers intended to provide breast milk for their infants. However, many preterm infants received a combination of formula and breast milk during their hospital admission. Detailed dietary intake data, via

either an enteral tube or bottle, were collected and calculated as per **Section 3.1.9**. A number of assumptions were necessary in these calculations: we relied on manufacturer's information regarding infant formula composition and we used a weekly EBM sample for macronutrient analysis and extrapolated the result to the entire week. It was sometimes not possible to collect an EBM sample, either due to the mother's low supply or logistical reasons, and this resulted in missing data. There were 148 (from approximately 830) occasions where EBM was not sampled equating to approximately 17% of the time. As a result of the assumptions and the missing data, we were cautious about applying inferential statistics to these data and present only descriptive statistics.

Macronutrient analysis of the EBM was done with the MilkoScan Minor™ (**Chapter 2**). The protein content of the sampled breast milk varied between 0.59 and 2.92 g/100mL with a mean of 1.27 g/100mL. Dietary intakes (excluding direct breast feeds) for the first four weeks of the study are described in **Table 4-7**. By week two, the median volumes in each group were similar and had increased to approximately 160 mL/kg/day; this is consistent with our feeding guidelines target of between 160 and 180 mL/kg/day.

Table 4-7 Protein, energy and volume of intake for the first 4 weeks of the study

	Higher protein (n = 43)	**Standard protein (n = 49)**
Study week 1		
Protein g/kg/day	4.1 (3.2 – 4.8)	3.6 (2.7 – 4.0)
Energy Kcal/kg/day	122 (90 – 145)	123 (94 – 141)
Volume mL/kg/day	146 (108 – 166)	152 (115 – 168)
Study week 2		
Protein g/kg/day	4.5 (4.0 – 4.9)	4.0 (3.4 – 4.3)
Energy Kcal/kg/day	140 (126 – 152)	143 (131 – 153)
Volume mL/kg/day	163 (146 – 172)	167 (156 – 174)
Study week 3		
Protein g/kg/day	4.2 (3.8 – 4.6)	3.7 (3.2 – 3.9)
Energy Kcal/kg/day	141 (128 – 152)	140 (128 – 153)
Volume mL/kg/day	164 (153 – 170)	166 (156 – 173)
Study week 4		
Protein g/kg/day	4.0 (3.4 – 4.5)	3.5 (3.2 – 3.7)
Energy Kcal/kg/day	138 (123 – 148)	140 (125 – 150)
Volume mL/kg/day	159 (145 – 168)	163 (154 – 169)

Values are median (interquartile range)

Intake data after study week 4 are not shown as the number of breast feeds increased significantly and displaced the measured intake. **Figure 4-2** shows the median volume of intake (mL/kg/day), and **Figure 4-3** the mean daily number of breast feeds, by group over the course of the study. Infants in the standard protein group had more breastfeeds after study week 4 and their measured enteral intake dropped accordingly.

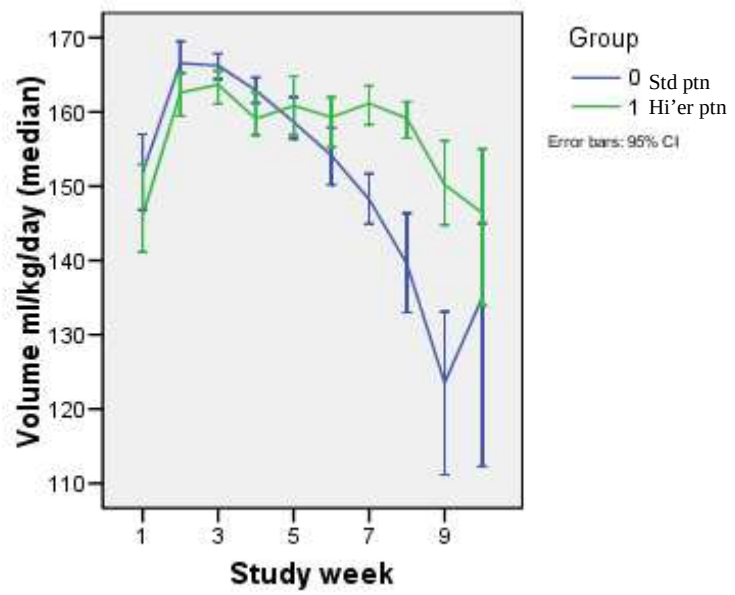


Figure 4-2 Volume of measured enteral intake during study

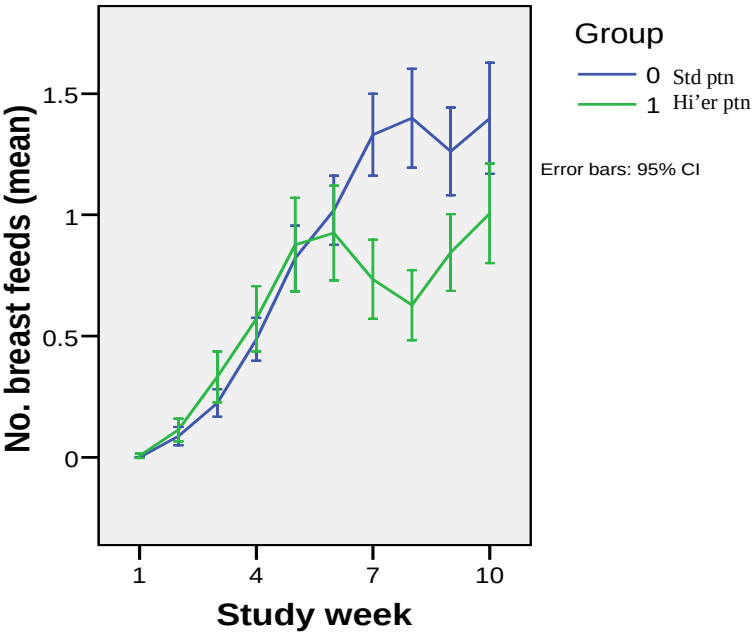


Figure 4-3 Mean number of daily breast feeds during study

4.4.4 Mode of Feeding

All study infants received at least some EBM as this was an inclusion criterion, but the proportion of EBM varied over the course of the study. **Figure 4-4** shows the proportion of breast milk in the diets of study infants at three different time points: the first week of the study, approximately midway through and at

discharge. Seventy three percent of infants received breast milk only in the first week of the study with the remainder receiving a mixed diet. By study week four, 62% of infants received breast milk only and 9% were fully formula fed, with the remainder of infants receiving mixed feeding. At discharge, 50% of infants were receiving breast milk only, with a further 26% having mixed feeds and 24% fully formula fed.

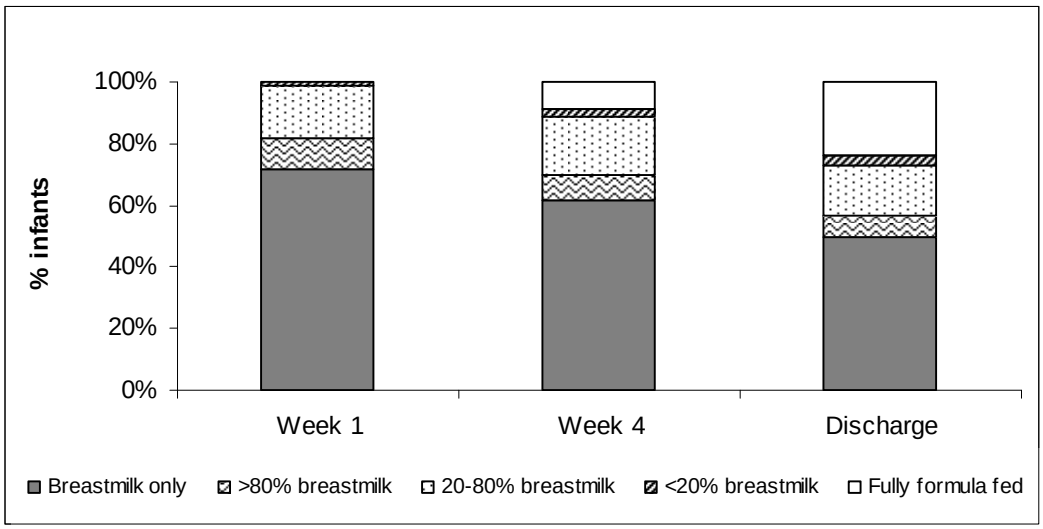


Figure 4-4 Breast milk as a proportion of measured enteral intake of infants during study

4.4.5 Summary of dietary intake and mode of feeding data

A feeding protocol was developed to ensure consistency for this trial. The documented nutritional management suggests that in practice, infants in the trial were slower than our protocol goals to commence trophic feeds, introduce HMF and reach full feeds. However, the nutritional management was not different between groups. The higher protein group received more protein from HMF than the standard protein group. The dietary intake data showed similar energy intakes between the two groups but higher protein intakes in the higher protein group, although no inferential statistics were performed on these data. The standard

protein group received more sucking feeds from the breast towards the end of the study than the higher protein group, and had a correspondingly lower measured enteral intake. Overall, approximately 75% of the infants received breast milk only in the first week of the study and this gradually declined with time to approximately 60% half way through the study and 50% at discharge.

4.5 Primary outcome: Length gain

4.5.1 Inter-observer variability study

An inter-observer variability study was conducted, as described in **Section 3.2**, to determine the technical error of measurement (TEM) between the author and each of the three research midwives involved in the anthropometric measurements. TEM is a measure of error variability, expressed in the same units as the measurement and is interpreted such that the measurement should come within ± the TEM two thirds of the time (178). The method used to calculate TEM is described in the WHO Multi-centre Growth Reference Study (179) and the following equation was used:

$$\textbf{TEM} = 1/N \sum_{i=1}^{N} \left[\sum_{j=1}^{2} Y_{ij}^{2} - \left(\sum_{j=1}^{2} Y_{ij} \right)^{2} / 2 \right]^{1/2}$$

Equation 4-1 Technical error of measurement, where N is the number of infants measured and Y_{ij} is one of the duplicate measurements taken by observer *j* for child ***i*.**

The TEMs recorded for length measurements for each of the three research nurses were 0.188, 0.243 and 0.202 cm. This compares favourably with other TEMs for length measurement reported in the literature which vary between 0.22 and 0.58 cm (179).

4.5.2 Length gain

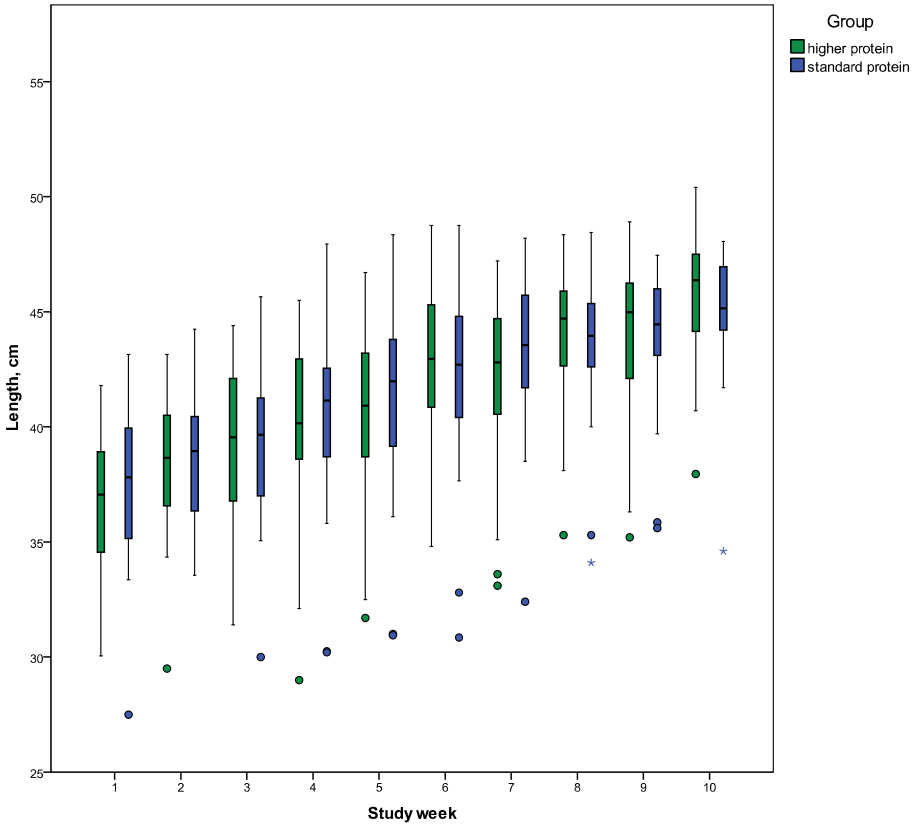


Figure 4-5 Unadjusted length for each group during the study
Boxes represent interquartile range with median represented by line; whiskers represent 1.5 x interquartile range, outliers (circles) are defined as points > 1.5 box-lengths from the edge of the box and * are extreme data points > 3 box-lengths from edge of box.

Figure 4-5 shows the unadjusted weekly length for all infants during the study.

The statistical model developed to analyse these data (see **Section 3.1.11**) uses the following GEE to estimate length:

*Length = 36.2015 + 0.1635*SD + 0.11*GroupA – 0.0078*SD*GroupA*

Equation 4-2. Estimated length of entire cohort of infants.
SD = Study day; GroupA = 1 if the infant is in the standard protein group; GroupA = 0 if the infant is in the higher protein group.

Diagnostic plots of the residuals were used to assess the fit of the model. For all three growth parameters the fit of the model was not consistent with the

assumptions of the model. It is likely that there is a variable which is not accounted for in the model which has an effect on growth in preterm infants. Possible explanations for this are given in the discussion.

The regression line of the mean length of the two groups adjusting for sex, gestational age and sibling clustering, is shown in **Figure 4-6**. Infants in the higher protein group grew at a slightly faster rate than those in the standard protein group. The length estimate of the higher protein group began a little below the standard protein group and increased at a slightly higher rate, to emerge above the standard protein group between study day 10 and 20. However, this trend was not enough to reach statistical significance (p = 0.08).

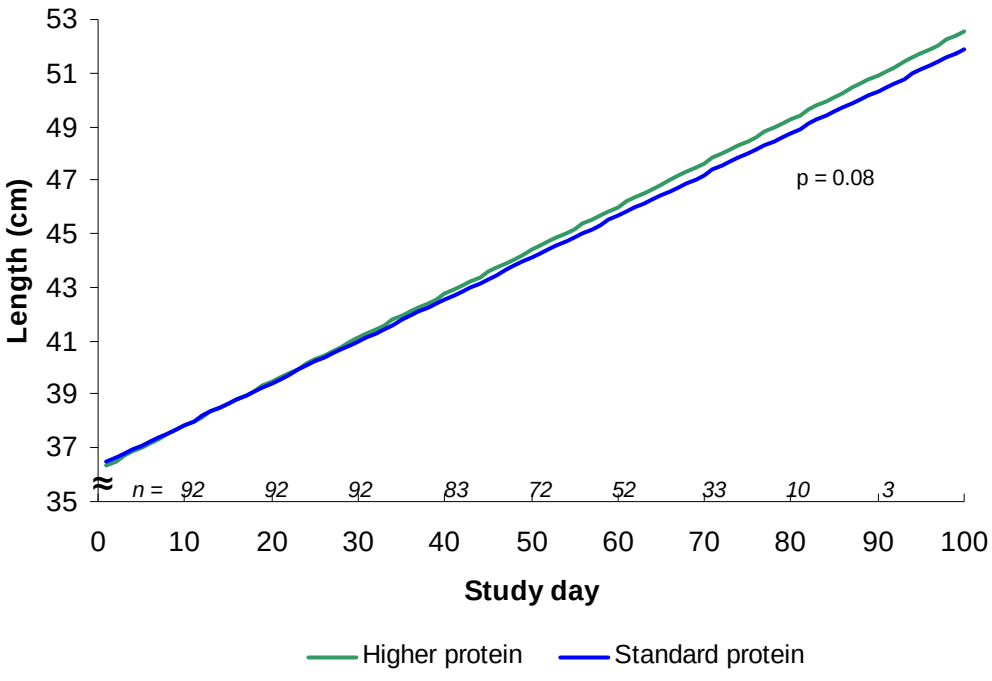


Figure 4-6 Estimated mean length of infants in each group over the study
n = number of infants still in study at each time point

Estimated weekly length gains, extrapolated from the graph, are described in **Table 4-8**.

Table 4-8 Estimated weekly length gain of study infants by group

	Higher protein (n = 43)	**Standard protein (n = 49)**	***P* value**
Length gain, cm/wk, (95% CI)	1.15 (1.1 – 1.19)	1.09 (1.05 – 1.13)	p = 0.08

4.5.3 Proportion of infants SGA for length

I was interested to see if the small non-significant weekly difference in linear growth translated to a difference in the classification of infants who were SGA, defined as <10th percentile according to Australian growth data (176, 177). This exploratory analysis is shown in **Table 4-9** and demonstrates a similar baseline incidence of SGA in the two groups. However, by discharge 63% of infants in the standard protein group were classified as SGA compared with 49% in the higher protein group, and this difference was statistically significant, following adjustment for GA and sibling clustering. For comparison, rates of SGA for length in preterm infants <33 weeks in the WCH neonatal unit were 13% at birth and 55% at discharge between 2001 and 2003 (33).

Table 4-9. SGA length status of study infants at birth and discharge by group

	Higher protein group (n = 43)	**Standard protein group (n = 49)**
SGA birth length, n (%)	7, (16)	7, (14)§
SGA discharge length, n (%)	21 (49)*	31 (63) §

§ n = 48; * p = 0.047, adjusted for sex, GA and sibling clustering

4.5.4 Compliance analysis

Secondary analyses for only the primary outcome were planned *a priori* for infants who complied with the protocol. Compliance was defined as those infants who received ≥70% of their measurable enteral intake as fortified breast milk over the course of the study (**Section 3.1.11**). There were two possible ways in which infants could not comply with the protocol. The first occurred when the infant was given formula, usually because the EBM supply was insufficient or, less commonly, because a special formula was clinically warranted. The other reason for non-compliance occurred when infants progressed to direct breastfeeds, which could not be fortified and were not measured. There were 29 and 30 infants who complied with the protocol in the higher and standard protein groups respectively. Seven pairs of twins were compliant and five pairs of twins were not compliant.

The equation used for estimating length from our model is:

Length = 36.7950 + 0.1637 SD -0.5578* GroupA - 0.0101* SD * GroupA.*

Equation 4-3. Estimated length of 'compliant' infants
SD = study day; Group A = 1 if the infant is in the standard protein group; Group A = 0 if the infant is in the high protein group.

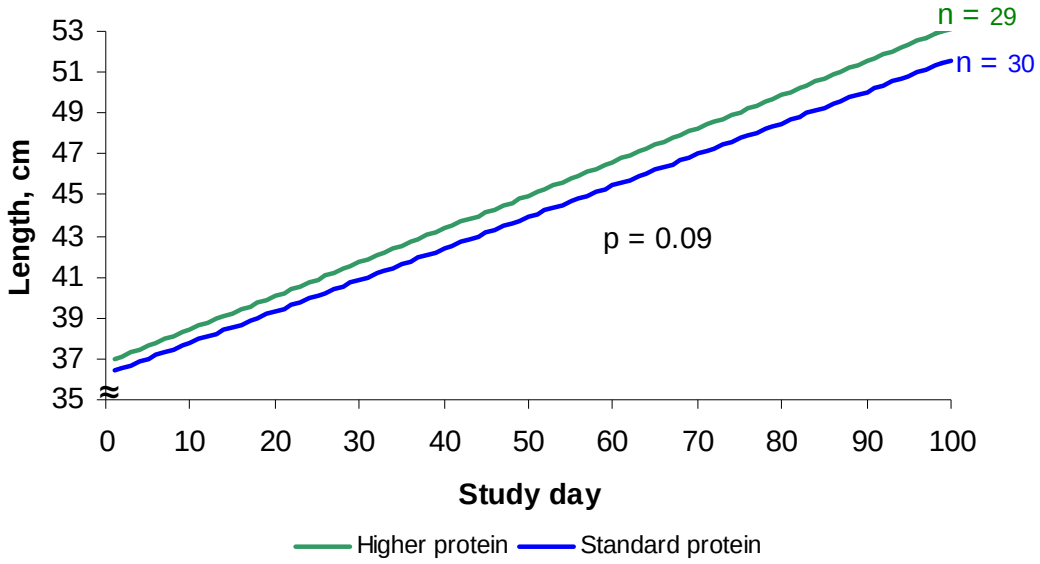


Figure 4-7. Estimated mean length of compliant infants by group

As shown in **Figure 4-7**, there is no statistically significant difference between the mean estimated lengths of the groups. Weekly estimated length gain is shown in **Table 4-10**.

Table 4-10 Estimated weekly length gain for compliant infants

	Higher protein (n = 29)	**Standard protein (n = 30)**
Length gain, cm/wk, (95% CI)	1.15 (1.09 – 1.21)	1.08 (1.02 – 1.13)

4.5.5 Length gain: Exploratory subgroup comparisons

Exploratory analyses, limited to the primary outcome of length gain, were performed *post hoc* for subgroups based on the stratification used in the randomisation schedule i.e. sex and GA. It is well established that both sex and GA affect growth and percentile charts have been developed incorporating these

variables (176, 177, 180). The same statistical model (GEE) was used for the subgroup analyses for consistency and comparability with the primary analysis.

4.5.5.1 Sex Subgroup

There were 19 males and 24 females in the higher protein group and 21 males and 28 females in the standard protein group. Four pairs of twins consisted of one male and one female, so these twins were separated for this subgroup analysis. The standard protein group contained 3 pairs of male twins, 4 pairs of female twins and 3 mixed twin pairs. The higher protein group contained 1 pair of male twins and 1 mixed twin pair. The equations used for estimating length from our model for females and males are:

Females

$$Length = 36.2818 + 0.1588*SD - 0.3087*GroupA - 0.0025*SD*GroupA$$

Equation 4-4. Estimated length of females

Males

$$Length = 35.9023 + 0.1687*SD + 0.7717*GroupA - 0.0138*SD*GroupA$$

Equation 4-5. Estimated length of males. SD = study day; GroupA = 1 if the infant is in the standard protein group; Group A = 0 if the infant is in the high protein group.

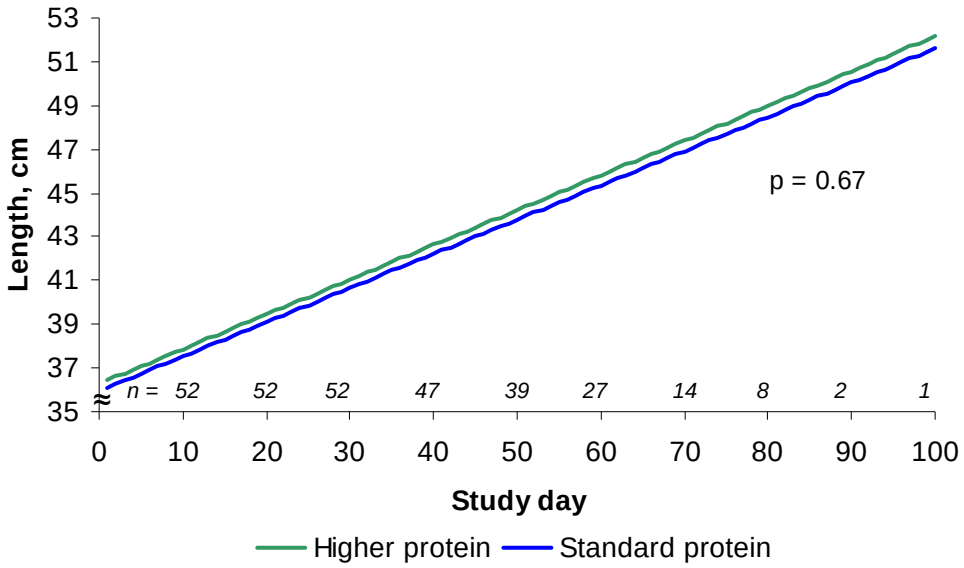


Figure 4-8. Estimated mean length, female subgroup
n = number of infants still in study at each time point

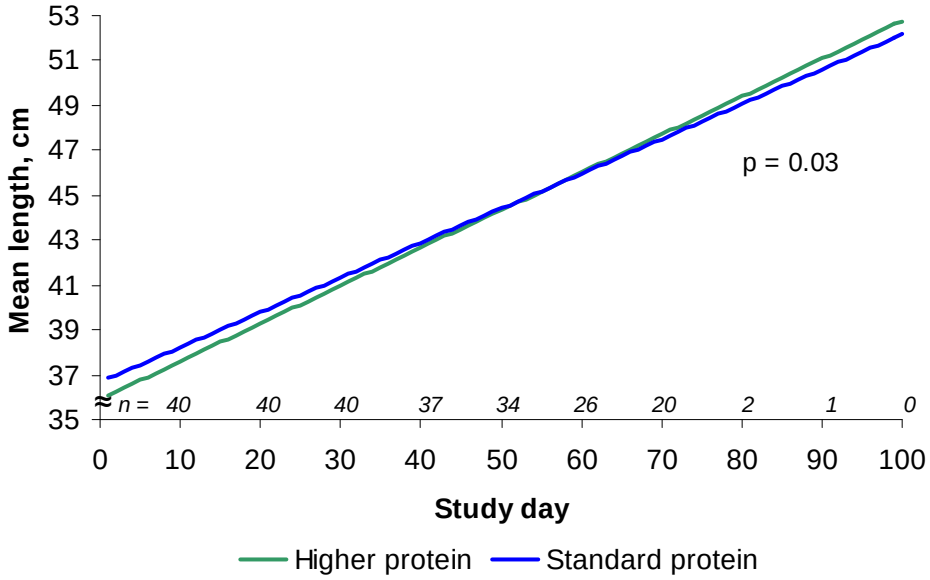


Figure 4-9. Estimated mean length, male subgroup
n = number of infants still in study at each time point

The estimated weekly length gains, extrapolated from the graph, are shown in **Table 4-11**.

Table 4-11 Estimated weekly length gain: Sex subgroup

	Higher protein	**Standard Protein**	***P* value**
Females, weekly length gain, cm, *(95% CI)*	1.11 (1.05 – 1.17) *n = 24*	1.09 (1.04 – 1.15) *n = 28*	p = 0.67
Males, weekly length gain, cm, *(95% CI)*	1.18 (1.13 – 1.24) *n = 19*	1.09 (1.02 – 1.15) *n = 21*	p = 0.03

There was no significant difference in growth between groups in the female infants (p = 0.67, Figure 4-8**),** whereas male infants grew faster if they were in the higher protein group compared to those in the standard protein group (p = 0.03, **Figure 4-9**).

4.5.5.2 Gestational age subgroup

The GA categories used for the subgroup analyses were the same as our stratification categories: i.e. <28 weeks and 28 – 30 weeks gestation. There were 23 infants born <28 weeks in the higher protein group and 20 in the standard protein group. Of the infants born <28 weeks gestation there were 1 pair of twins in the higher protein group and 4 twin pairs in the standard protein group. Infants born between 28 and 30 had 1 and 6 twin pairs in the higher and standard protein groups, respectively. The equations used for estimating length from our model are:

<28 weeks gestation

$$Length = 35.5019 + 0.1618*SD + 0.0583*GroupA - 0.0051*SD*GroupA$$

Equation 4-6. Estimated length of infants born <28 weeks

28 – 30 weeks gestation

$$Length = 35.4707 + 0.1697*SD + 0.6372*GroupA - 0.0153*SD*GroupA$$

Equation 4-7. Estimated length of infants born 28 - 30 weeks. SD = study day; GroupA = 1 if the infant is in the standard protein group; Group A = 0 if the infant is in the high protein group.

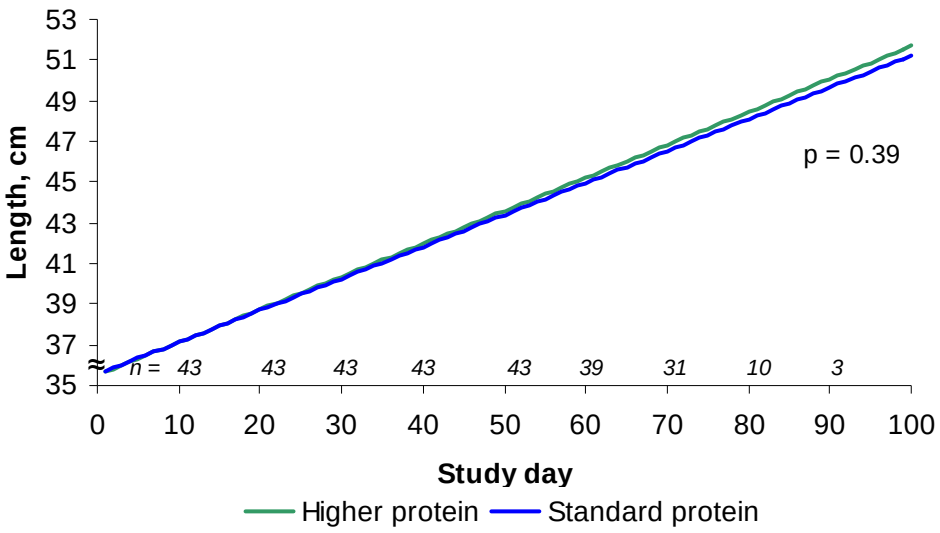


Figure 4-10. Estimated length of infants born <28 weeks gestation
n = number of infants still in study at each time point

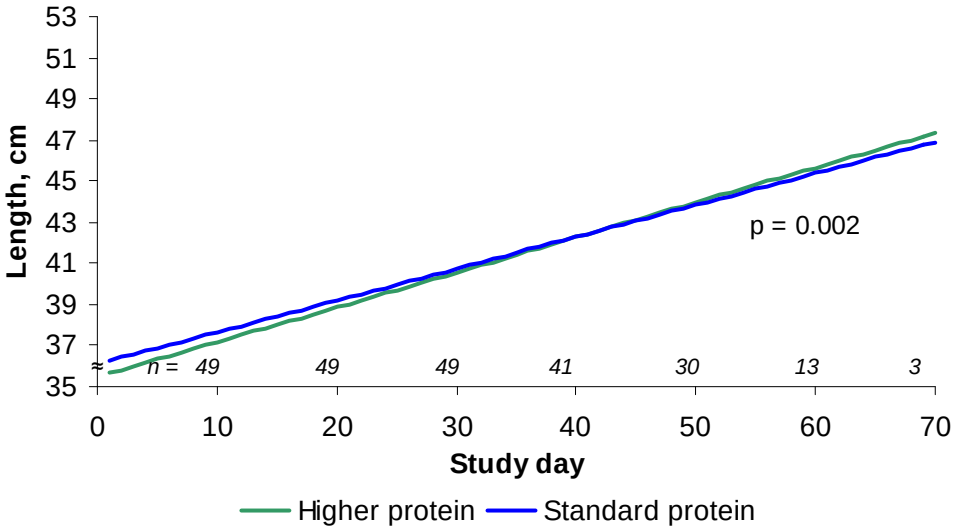


Figure 4-11. Estimated length of infants born 28 - 30 weeks gestation
n = number of infants still in study at each time point

Figure 4-10 demonstrates no significant difference between the groups in the length gain of the infants born <28 weeks gestation (p = 0.39). However, for infants born 28 – 30 weeks gestation **(Figure 4-11**), length gain was greater in the higher protein group (p = 0.002).

Table 4-12. Estimated weekly length gain: GA subgroup

	Higher protein	**Standard Protein**	***P* value**
<28 weeks gestation, weekly length gain, cm, *(95% CI)*	1.13 (1.08 – 1.19) *n = 23*	1.1 (1.03 – 1.12) *n = 20*	p = 0.39
28 – 30 weeks gestation, weekly length gain, cm, *(95% CI)*	1.19 (1.15 – 1.23) *n = 20*	1.08 (1.03 – 1.13) *n = 29*	p = 0.002

Estimated weekly length gain in infants born <28 weeks gestation was very similar (1.13 and 1.1 cm/week in the higher and standard protein groups, respectively). Infants born 28 – 30 weeks gestation had estimated weekly length gains of 1.19 and 1.08 cm/week in the higher and standard protein groups, respectively (**Table 4-12).**

4.6 Secondary efficacy outcomes

4.6.1 Weight gain

Figure 4-12 shows the mean estimated weight for each group plotted against study day, using the GEE model adjusting for gender, gestational age and sibling clustering. The equation for estimating weight is:

*Weight = 113.7.418 + 23.7845*SD – 118.12*GroupA + 2.1692*SD*GroupA*

Equation 4-8. Estimated weight SD = Study day; GroupA = 1 if the infant is in the standard protein group; GroupA = 0 if the infant is in the higher protein group.

Infants in the standard protein group started at a lower weight but increased more rapidly than the higher protein group, such that their mean weight overtook the higher protein group at around study day 50 and then continued to rise slightly above it. However, this difference is not statistically significant (p = 0.33).

Table 4-13 shows the average daily weight gain of infants (g/day) of the two groups.

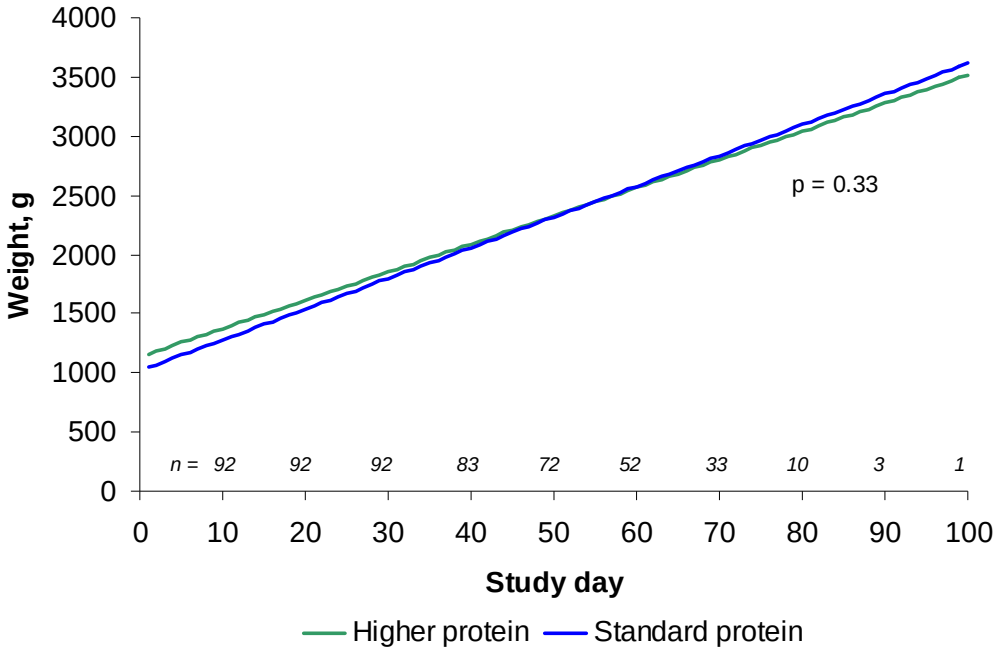


Figure 4-12. Estimated mean weight of study infants
n = number of infants in the study at each time point

Table 4-13. Estimated mean daily weight gain of study infants

	Higher protein (n = 43)	**Standard protein (n = 49)**	***P* value**
Weight gain, g/day, (95% CI)	24 (20 – 28)	26 (24 – 28)	p = 0.33

4.6.2 Proportion of infants SGA for weight

SGA status was defined using Australian data for twins and singletons (176, 177). *Post hoc* analysis is shown in **Table 4-14** and demonstrates a similar incidence of SGA at birth, with a rise in the number classified as SGA at discharge but no difference between groups. For comparison, rates of SGA for weight in preterm infants <33 weeks in the WCH neonatal unit were 8% at birth and 32% at discharge between 2001 and 2003 (33).

Table 4-14. SGA weight status of study infants at birth and discharge

	Higher protein group (n = 43)	**Standard protein group (n = 49)**
SGA birth weight, n (%)	7 (16)	6 (12)
SGA discharge weight, n (%)	15 (35)	17 (35)

4.6.3 Occipital head circumference

The equation used for estimating OHC from the GEE model is;

$$OHC = 25.6560 + 0.1341*SD - 0.1317*GroupA + 0.0022*SD*GroupA$$

Equation 4-9. Estimated occipital head circumference

SD = Study day; GroupA = 1 if the infant is in the standard protein group; GroupA = 0 if the infant is in the higher protein group.

Figure 4-13 shows the mean OHC of the two groups, using the model adjusted for sex, gestational age and sibling clustering. There is no significant difference in OHC growth between the two groups.

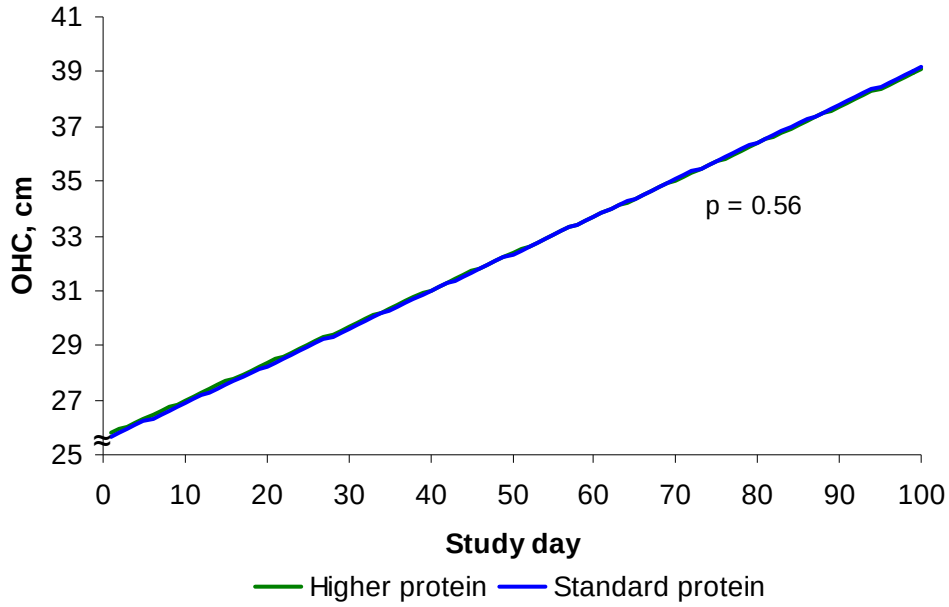


Figure 4-13 Estimated mean occipital head circumference of study infants

Table 4-15. Estimated mean weekly OHC growth of study infants

	Higher protein (n = 43)	**Standard protein (n = 49)**	***P* value**
OHC**,** cm/week, (95% CI)	0.94 (0.9 – 0.98)	0.95 (0.92 – 0.99)	p = 0.56

4.6.4 Proportion of infants SGA for head circumference

SGA status was defined using Australian data for twins and singletons (176, 177). The results of this *post hoc* analysis are shown in **Table 4-16**. As with the other growth parameters reported, the incidence of SGA increases between birth and discharge but there is no difference between groups in the proportion of infants classified as SGA at birth or discharge. For comparison, rates of SGA for OHC in

preterm infants <33 weeks in the WCH neonatal unit were 7% at birth and 11% at discharge between 2001 and 2003 (33).

Table 4-16. SGA OHC status of study infants at birth and D/C

	Higher protein (n = 43)	**Standard protein (n = 49)**
SGA birth OHC, n (%)	3 (7)	5 (10)
SGA discharge OHC, n (%)	8 (19)	11 (22) §

§ n = 48

4.7 Discussion

This trial was designed as a pragmatic trial to test the efficiency, rather than the efficacy, of a higher protein fortifier. In clinical trials an efficacy trial is designed to test an intervention under optimal conditions, whereas an efficiency trial is designed to test an intervention under 'real world' conditions (181). For example, ideal conditions for an efficacy trial of human milk fortifiers would be created if the infants were selected to be very similar to each other, they were fed only fortified breast milk (no direct breastfeeding or infant formula) and the breast milk was of uniform composition. However, these conditions do not reflect the current feeding practices of neonatal units and this trial was designed to test a higher protein fortifier in a real life situation.

This RCT aimed to increase the protein content of fortified EBM to levels at least equivalent to current preterm infant formula. A concern regarding the trial was that the small difference in protein concentration of the trial fortifiers may be lost

in the large variability of protein intakes due to the use of infant formula and the variation in the protein content of the EBM. However, **Table 4-6** shows that a mean additional 0.8 g protein/day was provided to the higher protein group via the fortifier. The fortifiers used in the trial differed in protein content by 0.4 g protein/100mL EBM and were isocaloric. Therefore, at feeding volumes of approximately 160 mL/kg/day, we would expect to see a difference of approximately 0.6 g/kg/day in protein intake with no difference in energy intake. Indeed, despite the assumptions and missing data associated with the dietary intake data **Table 4-7** indicates a higher median protein intake of 0.5 g/kg/day in the higher protein group but similar energy intakes for the first 4 weeks of the study. It therefore appears that the intervention was successful even in the mixed feeding environment which is typical of modern Australian neonatal units, at least for the first 4 weeks of the study. After this time, it was not possible to obtain accurate measures of total dietary intake as the infants commenced direct breastfeeding. The frequency of non-fortified breast-milk feeding was greater in the standard protein group **(Figure 4-3)** and this would result in a lower protein intake. At the same time, the proportion of infants fed human milk fell from 73% in the first week to 62% in the 4th week (**Figure 4-4**) therefore increasing the number of infants fed infant formula. These confounding factors introduced heterogeneity both within and between the groups as the study progressed. **Figure 4-6** shows the primary outcome variable, length, over the course of the study, by treatment group. It appears from this figure that there is very little difference in length between treatment groups in the first 4-5 weeks of the study when intakes were less confounded, and differences appear to emerge after this time, coinciding with the increased number of breast feeds in the standard protein group. It is

possible that the difference in growth reflects the negative effect of greater frequency of direct breastfeeds on protein intake in the standard protein group (**Figure 4-3)** rather than the positive effect of the higher protein fortifier. However, if this were the case, there would also be a difference in energy intake and therefore an expected difference in weight gain between the groups. This was not the case. It could also equally be argued that the higher protein group, which consisted of more immature infants (one infant born at 23 weeks gestation and 5 at 24 weeks, **Section 4.3.3**), may have taken longer to respond to the intervention with differences in length gain becoming apparent later in the study. However, this trial was not designed to look at efficacy over the first 4-5 weeks of the study and is not powered to detect the differences expected over this time. Hence, the conclusions drawn are based on the original aims of this trial, which was to test the efficiency, rather than the efficacy of a higher protein fortifier from the start of fortification to discharge or EDD, whichever came first.

My RCT demonstrated that a higher protein fortifier slightly improved the length gain of infants but not enough to reach statistical significance using the conservative GEE analyses. We chose the conservative analyses to account for the imbalance of twins between the groups. However, the statistical model used was less stable than we would have liked as assessed by the fit of the model using diagnostic plots of the residuals (**Section 4.5**). The imbalance of twins in the study may at least partly account for this lack of fit of the model. Many of the twins were a result of IVF conception (7 out of 12 sets of twins) and these infants may be different to other infants in the study in some way. The clustering adjustment used in our statistical model may not fully account for the fact that

twins were randomised as a single unit. Nevertheless, despite these issues, the effect size is not inconsistent with other studies where, in general, there have been small improvements in length gain which have either been established (150) or not reached statistical significance (151, 152). The only contrasting result has been from Sankaran *et al* (149) who showed improved growth (not significant) with a lower protein liquid fortifier. However, this study compared a liquid with a powdered fortifier and so is not directly comparable with the other studies.

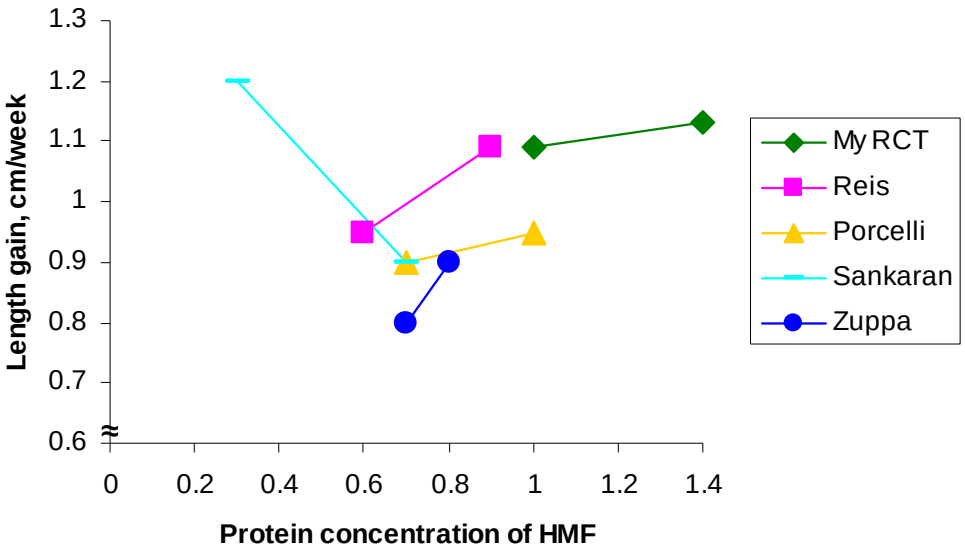


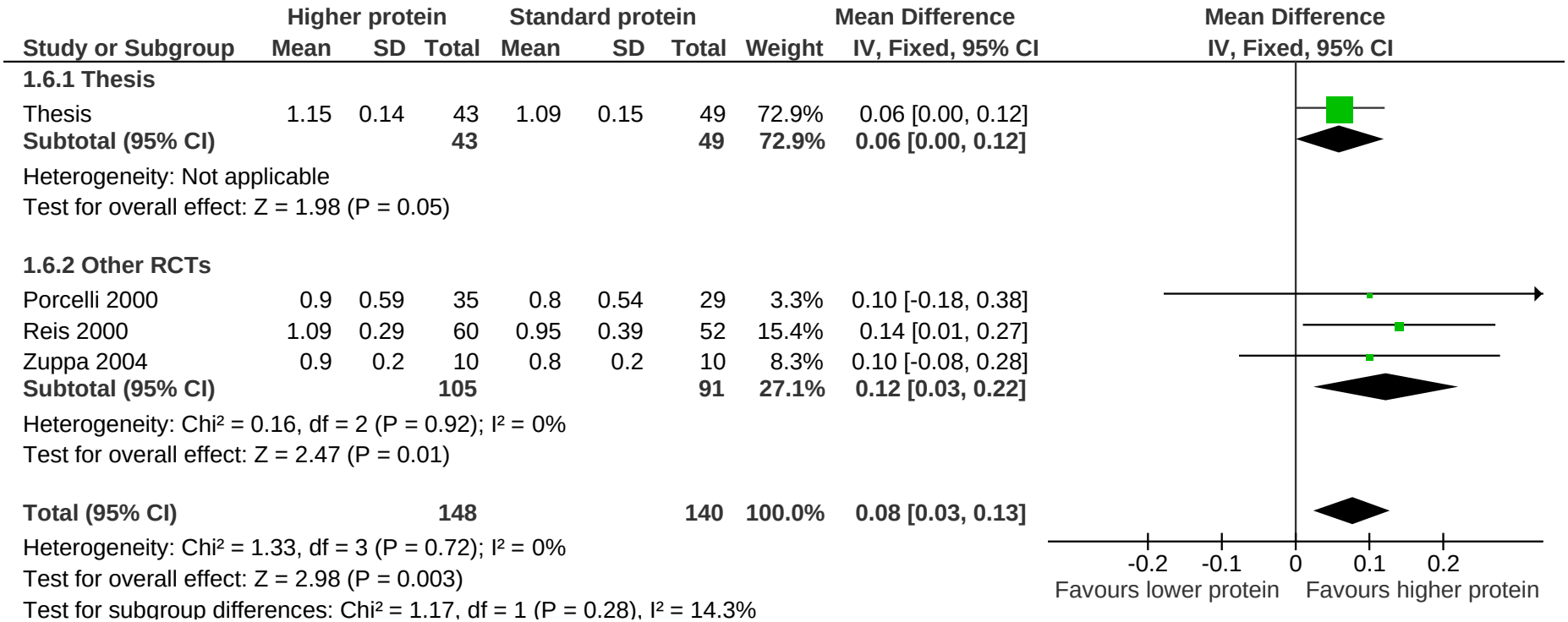
Figure 4-14 Comparison of trials: length gain in response to protein concentration of HMF

Figure 4-14 compares previous HMF trials and shows the change in length gain with differing protein concentrations. My RCT is unique in that the control level of protein is equivalent to the higher level used in previous trials. Control levels of protein used in previous trials are relatively low compared to our current knowledge about requirements (28, 56) and it is possible that the larger effect size seen in these studies is due to the intervention increasing the protein intake from inadequate to adequate. By contrast, my RCT compares higher levels of protein

fortification and it is possible that manipulations in protein content at higher levels may result in a smaller effect size. Furthermore, my trial included infants born ≤30 weeks gestation, whereas previous trials included more mature infants (149-151) and this may have also affected the growth pattern.

To facilitate comparison, I have combined the length gain data from my clinical trial with previous comparable HMF trials, using different protein concentrations while keeping energy constant, in a meta-analysis. Length gain data were extracted from 3 other trials (150-152) and included a total of 288 infants. **Figure 4-15** indicates there is a small growth advantage to increased protein with a difference in means of 0.08 cm/week (CI 0.03 – 0.13, p = 0.003). The heterogeneity of the subgroup of RCTs other than my thesis is low ($Chi^2 = 0.16$, df = 2, p = 0.72, $I^2 = 0$). However, the subgroup difference between my trial and other trials, shows an increase in heterogeneity ($Chi^2 = 1.17$, df = 1, p = 0.28, $I^2 = 14.3$). My trial may not be directly comparable with the other studies because the control level of protein in my study was equivalent to the treatment level of earlier studies.

Figure 4-15. Meta-analysis of length gain of preterm infants in trials of HMF with differing protein but same energy concentrations



IV = inverse-variance

Arslanoglu *et al* (127) compared a fixed fortification regime (adding 0.8 g protein) with an adjusted regime based on urea nitrogen levels. If SUN levels fell below designated cut offs, the fortification level was increased using more HMF in combination with a protein powder. The '+2' level of adjusted fortification provided an additional 1.4 g protein, equivalent to my higher protein group, and the '+3' level an additional 1.8 g protein. Despite the use of these high levels of fortification, dietary protein intakes of the adjusted regime group were less than intakes in my standard protein group (week 1: 2.9 vs 3.6; week 2: 3.2 vs 4.0; week 3: 3.4 vs 3.7 g/kg/day for Arslanoglu and my trial, respectively). Both trials measured the protein content of the EBM using equivalent methods, so measurement differences would not account for the discrepancy. However, infants in the trial of Arslanoglu *et al* were fed exclusively human milk, and donor milk, which is characteristically low in protein, was used if there was a deficit, resulting in dietary intakes lower than generally assumed (147). In contrast, my trial used preterm infant formula when EBM supply was insufficient, resulting in higher protein intakes. Arslanoglu *et al* showed improved weight gain (17.5 ± 3.0 vs 14.4 ± 3.0 g/kg/day, $p < 0.01$, adjusted vs fixed regimes, respectively) and head circumference gain (1.4 ± 0.3 vs 1.0 ± 0.3 cm/week; $p < 0.05$) using the adjusted regime. While length gain did not reach statistical significance (1.3 ± 0.5 vs 1.1 ± 0.4 mm/day $p > 0.05$), the effect size is larger than that seen in the meta-analysis. Energy intakes were not statistically significantly different between the two groups, which is surprising as extra HMF and protein would supply extra energy. As with the previous studies discussed, the intervention in Arslanoglu's trial may have been enough to move the protein intake from the category of inadequate to adequate and consequently show a larger effect size. This study highlights the

complexity of fortifying milk and the difficulty of a 'one fits all' approach with commercial fortifiers. The choice of donor human milk or preterm formula to supplement mother's own EBM has implications for the amount of protein required in the fortifier.

Other growth parameters (weight and OHC gains) showed no significant difference between groups in my trial. This is in contrast to previous trials which have shown increased weight gain with the higher protein group (150, 151). A possible explanation for this anomaly is that we chose to report weight gain in g/day (adjusted for GA), rather than use g/kg/day. The higher protein group included 5 of the least mature and smaller infants who had smaller gains. The outcome of weight gain/day is a ratio variable, which when divided by another variable (weight) creates a ratio divided by a ratio and may lead to incorrect conclusions about the relationships between the outcome and the variable (182). The standard protein group also contained more twins who tend to have greater catch-up growth which is more evident in weight gain than length gain. It is possible that adjustment for clustering did not account for the influences of twinning.

Studies included in the meta-analyses, including my RCT, measured growth during the study period, rather than from birth. The definitions used for the study start varied but all defined it as either an enteral intake >100 or more, mL/kg/day or the start of fortification (150, 151), although one study did not define the start (152). My RCT defined the study start as commencement of fortification and this occurred at a mean enteral intake of 120 mL/kg/day. This volume provides an

energy intake of 100 Kcal/kg/day which is sufficient to support growth. An alternative approach to the analyses of my RCT would have been to measure growth from birth rather than from the start of the intervention, thereby describing growth over the entire hospital admission. However, this would have included the inadequate protein and energy intakes in the very early period after birth, before feeding was fully established.

While we chose to focus specifically on growth during the intervention, the difference in SGA classification from birth to discharge gives a rudimentary description of growth over the hospital admission period. This *post hoc* exploratory analysis showed a substantial increase in the SGA length classification at discharge in both groups, with a baseline incidence at birth of approximately 15%, rising to 49% and 63% in the higher and standard protein groups, respectively. As previously mentioned, the rate of SGA for length at discharge in the WCH neonatal unit has been documented at 55% for infants born <33 weeks gestation and 77% for infants born <28 weeks gestation. This would indicate that there is room for improvement in length growth in all these infants, but that the higher protein fortifier did appear to ameliorate the incidence of SGA for length at discharge. However, it must be acknowledged when interpreting these results the issues confounding the nutrition intervention in the latter part of the study, as discussed above. The number of infants classified as SGA for both weight and head circumference also increased between birth and discharge but both groups had similar increases. Approximately 35% of infants in both groups were SGA for weight at discharge and 19 and 22% of infants were SGA for head circumference in the higher and standard protein groups, respectively. Poor length

growth (stunting) in infancy and early childhood has been found to be a strong predictor of poor neurodevelopmental outcomes (183-186) and long term economic outcomes (184). However, much of this literature relates to term infants in developing countries where lack of schooling or poor environment may play a role. The clinical significance of stunting in preterm infants is less clear, although it would seem logical that malnutrition severe enough to affect growth at such a vulnerable developmental time would have consequences. Preterm infants are known to have poorer neurodevelopmental outcomes at school age and beyond compared to infants born at term (45, 47, 187-189).

In the exploratory analyses, length gain was consistently greater in the higher protein group but this only reached statistical significance in males and those born 28 – 30 weeks gestation. Cooke *et al* (11) found that infants fed a high protein formula (3.6 g/100 Kcal) gained weight at a faster rate than those fed formula containing 3.0 g/100 Kcal and that boys gained at a faster rate than the girls (mean difference of 10g/day). For comparison, these protein energy ratios are higher than the fortified milk in my trial which had protein energy ratios of 3.2 and 2.7 g/100Kcal (higher and standard protein groups, respectively), calculated using the mean protein value for EBM in my cohort of 1.27 g/100mL. Cooke *et al* postulated that boys are programmed to grow faster than girls with both fetal (180) and postnatal (29) growth rates being greater in boys than girls. Therefore, in conditions where their requirements are supplied, boys' gains would tend to be greater than girls'. Cooke did not report length gain in his short term nutrient balance but it would be reasonable to assume that the same argument may apply to

length gain. However, in my trial, it is possible that the effect found in males is due to chance because of the small number of infants.

A significant effect of higher protein treatment was also found in the subgroup of infants born 28 to 30 weeks' gestation. Other studies have not reported length gain in gestational age subgroups so it is not known if this is a common finding. However, this subgroup of my trial corresponds closely to the cohort of infants in the Reis trial (150) as they were more mature (≤ 33 weeks gestation) with a mean gestational age of 29 weeks. A similar effect size was seen in my subgroup and the Reis trial (length gain difference of 0.11 and 0.14 cm/week respectively). There are multiple clinical factors which can affect growth and not surprisingly, the infants born <28 weeks gestation are most at risk of a complicated clinical course. In my trial, there was more NEC (14% vs 4%), sepsis (21% vs 8%), surgery (47% vs 12%) and oxygen requirement at 36 weeks of age (60% vs 22%) in the less mature infants. The combination of these factors may increase variability and compound to limit the dietary intake of the most immature infants. The failure to respond to the intervention in the less mature subgroup may also be explained by further examination of their dietary intakes. The less mature infants took longer to grade up to full feeds than the more mature infants (mean (SD) 22 (11) days: 14 (6) days respectively) and consequently, protein and energy intakes were lower in the first week of the study (3.3 (1.3) vs 3.8 (1.1) g protein/kg/day and 105 (40) vs 122 (34) Kcal/kg/day in the <28 week and 28-30 week groups, respectively). These infants may have accrued a greater protein and energy deficit than the more mature infants and intakes may have been below requirements. The significant effect of the intervention seen in the more mature infants may be due to

less heterogeneity of the group and better meeting their requirements throughout the whole study period. However, it is difficult to know if this is the case or whether the differences in subgroups may be due to chance because of the lack of significant interaction by GA strata.

In conclusion, my RCT has shown a small, although non-significant, effect of increasing the protein content of HMF on the linear growth of preterm infants. In secondary analyses, these small weekly differences in growth translated to a difference in SGA classification for length at discharge. The high overall incidence of SGA at discharge indicates that there is room for improvement in growth for preterm infants. Unlike some previous trials in this area, my trial included infants who were supplemented with formula, and so is generalisable to the usual infant population seen in preterm nurseries.

Chapter 5 Safety Results

5.1 Introduction

One of the barriers to increasing the protein concentration of fortifiers is the risk of excessive protein intakes in very preterm infants. Early studies using very high protein intakes for infants (above 6 g/kg) have been associated with adverse effects such as lethargy, fever and poor feeding (88) as well as high blood levels of some amino acids (156). Very preterm infants have immature organs and immature metabolic pathways and their ability to digest protein, metabolise amino acids and excrete nitrogen may be limited. Adding to this issue is the unknown and highly variable protein content of EBM (115, 119, 125, 159). Individualised fortification regimes that rely on either measuring the protein content of the milk before fortification (126), or adjusting fortification based on the biochemical response of the infant (127, 128) have been proposed as a solution to this problem. However, Arslanoglu *et al* (127) found that almost all of the adjustments made to the fortification regime were an increase rather than decrease in the amount of fortifier added, suggesting that concerns about protein overload may be overstated. Conversely, low protein intakes are associated with hypoproteinaemia and oedema (62). Consequently, it was important in my trial to monitor renal function as well as other biochemical markers of protein nutritional status and metabolism.

The use of HMF has been implicated in gastrointestinal tolerance of feeds due to the increased osmolality of the milk (190) and a possible increased incidence of

NEC (72), although this trial lacked the power to detect a significant difference. Other clinical outcomes such as length of hospital stay and PMA at discharge may also be influenced by increasing the protein content of the diet. Thus it was necessary to report the incidence of these events in my trial.

The hypothesis was that increasing the protein content of the fortifier would result in higher biochemical markers of protein metabolism but no increase in biochemical disturbances or adverse events. The primary safety outcomes were SUN, plasma albumin, creatinine and blood pH and amino acid levels. Secondary safety outcomes were the clinical outcomes of the infants.

5.2 Statistical Analyses

The secondary biochemical outcomes (urea, creatinine, albumin and pH) were analysed by a DMAC statistician using the same model used for the primary outcome and is described in more detail in **Section 3.1.11**. Briefly, differences between groups over time were assessed with a repeated measures model using GEEs to adjust for variance for clustering on siblings and measures within infants over time. The model included the fixed effects: intercept, study day (SD) and the treatment. The model also adjusted for sex and GA. Blood amino acids were also analysed by the DMAC statistician using the same model as used for urea, creatinine, albumin and pH. However, there was no interaction between time and the treatment group and time was not significant in the model. Therefore the data was summarised and the least square of the means was used to compare groups. The model included GA, sex and sibling clustering.

Gastrointestinal and respiratory outcomes, ROP and other clinical outcomes described in **Section 5.5.4** were analysed by the author using PASW Statistics (Version 18.0.0, Chicago, Il, USA). Independent sample T test was used for normally distributed continuous variables and Chi-squared test for categorical variables with Fischer's exact test used for variables with a low incidence.

5.3 Biochemical markers of protein metabolism

Blood tests were initially performed weekly and then twice weekly when the infants were nearing discharge, and analysed for serum urea, plasma creatinine, albumin, blood pH and amino acids as described in **Section 3.4**.

5.3.1 Urea

Figure 5-1 shows the unadjusted weekly mean serum urea levels in the trial.

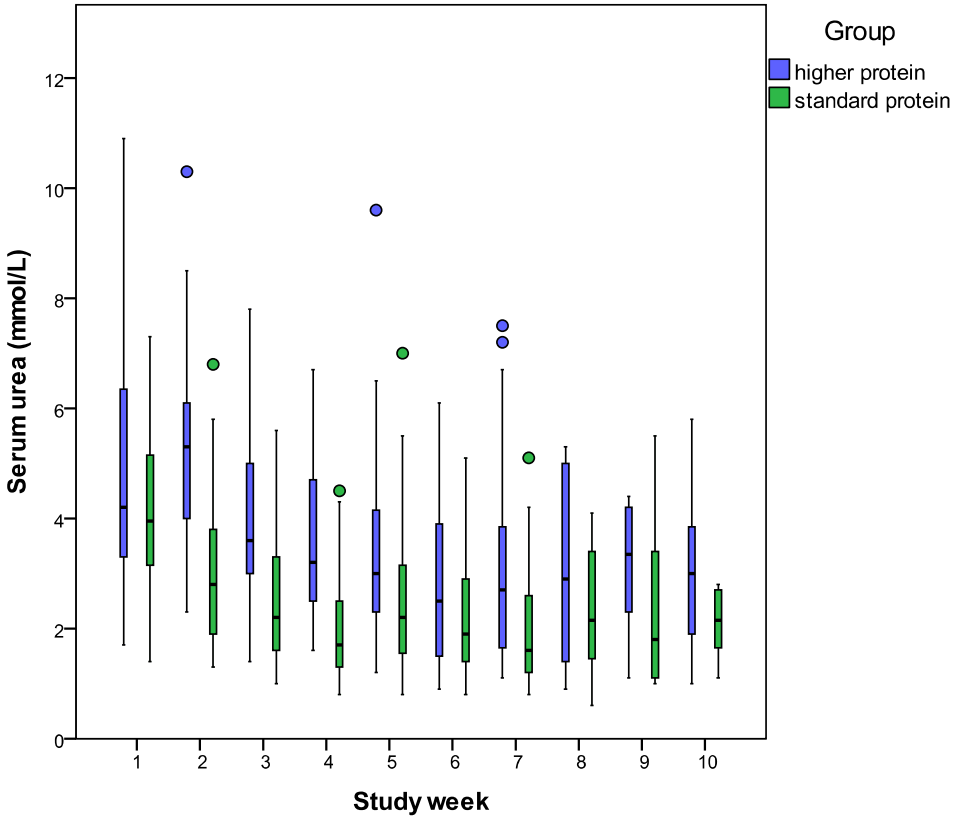


Figure 5-1 Unadjusted weekly mean serum urea levels during the trial
Boxes represent interquartile range with median represented by line; whiskers represent 1.5 x interquartile range, outliers (circles) are defined as points > 1.5 box-lengths from the edge of the box

After discussion with neonatologists, and for the purposes of this study population, we defined uraemia as SUN levels above 8 mmol/L. Other researchers have used different definitions. Cooke *et al*, in a study on high protein preterm infant formula, used >7 mmol/L to define uraemia (191). Arslanoglu *et al* ,in their adjustable fortification regime, did not define uraemia but used 5 mmol/L as the level at which they reduced the amount of fortifier added (127). Porcelli et al (151) defined the upper limit of the normal range as 28 mg/dl (10 mmol/L). In

the RCT reported in this thesis there were seven infants (all from the higher protein group) who developed transient uraemia (SUN >8 mmol/L), and these all occurred within the first 10 days of the study. The highest level recorded was 10.9 mmol/L. All urea levels resolved spontaneously without any intervention. Conversely, 15 infants (on 22 occasions) recorded SUN levels below the normal reference range (≤1 mmol/L), indicative of poor nutritional status. Eleven of the fifteen infants were in the standard protein group. Similarly, these events occurred early in the study, before day 9.

In the overall analysis, the equation used for estimating urea was:

$$Urea\ (mmol/L) = 4.776 - 0.025 * SD - 1.131 * Group\ A$$

Equation 5-1. Estimated urea levels, where SD = study day, Group A = 1 if the infant is in the standard protein group and Group A = 0 if the infant is in the higher protein group

Figure 5-2 shows the adjusted urea levels during the study for the two groups. Urea significantly reduced over the course of the study, independent of treatment group ($p < 0.0001$). Infants in the higher protein group had significantly higher urea levels ($p < 0.0001$) compared with the control over the duration of the study.

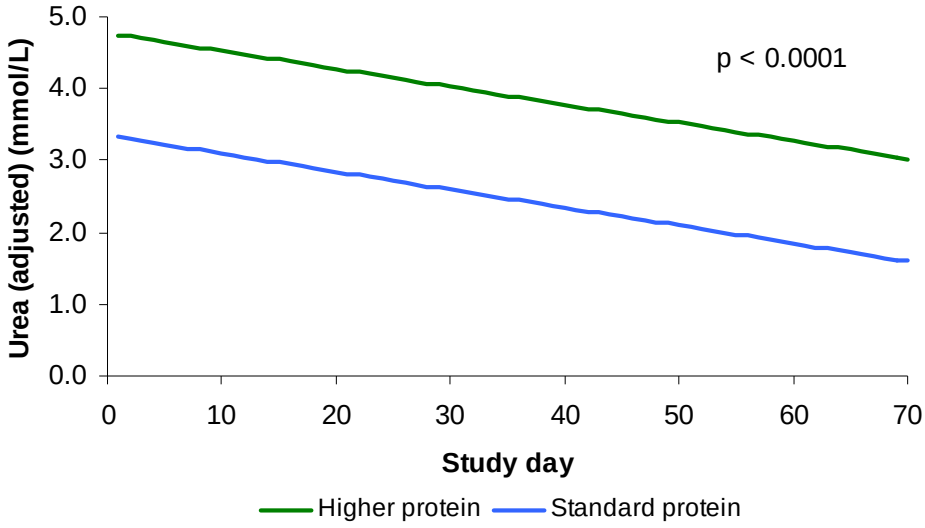


Figure 5-2 Adjusted serum urea concentrations during the study

5.3.2 Plasma creatinine

Unadjusted weekly mean creatinine levels are shown in **Figure 5-3** and it can be seen that there is variability in these data and also considerable overlap between the two treatment groups.

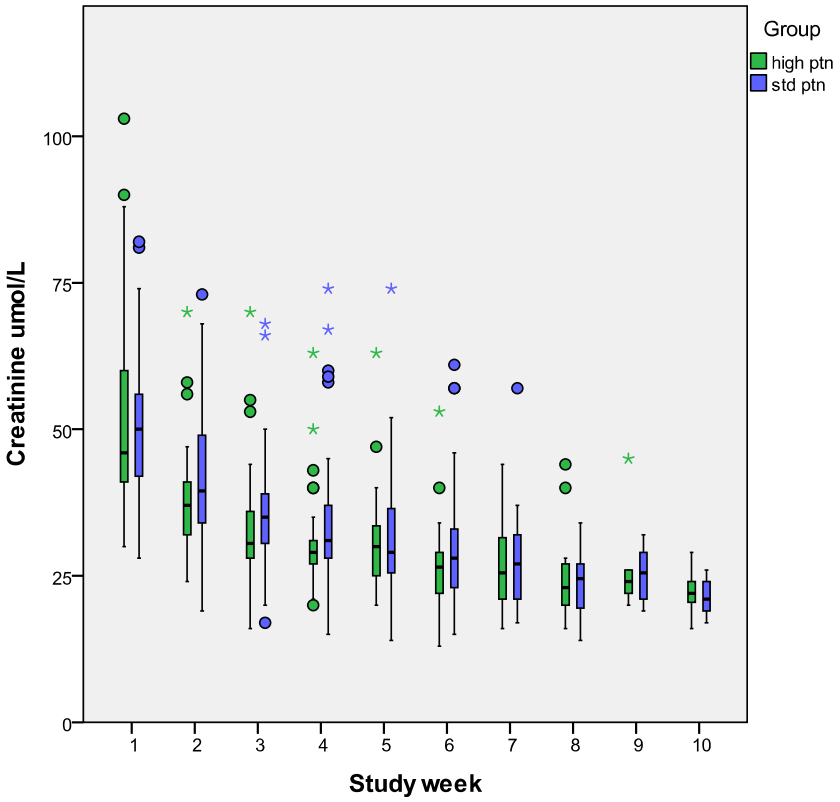


Figure 5-3 Unadjusted weekly creatinine levels
Boxes represent interquartile range with median represented by line; whiskers represent 1.5 x interquartile range, outliers (circles) are defined as points >1.5 box-lengths from the edge of the box. Extreme values (*) are >3 IQR from end of box.

The equation used for estimated the creatinine was:

$$Creatinine\ (\mu mol/L) = 45.31 - 0.39 * SD + 0.93 * groupA$$

Equation 5-2 Estimated creatinine levels, where SD = study day, Group A = 1 if the infant is in the standard protein group and Group A = 0 if the infant is in the higher protein group

Adjusted plasma creatinine levels, using the model described in **Section 5.2**, are shown in **Figure 5-4**. Similar to serum urea levels, there was a significant

decrease in levels over time independent of treatment group ($p < 0.0001$). There was no significant difference in plasma creatinine levels between treatment groups ($p = 0.6$).

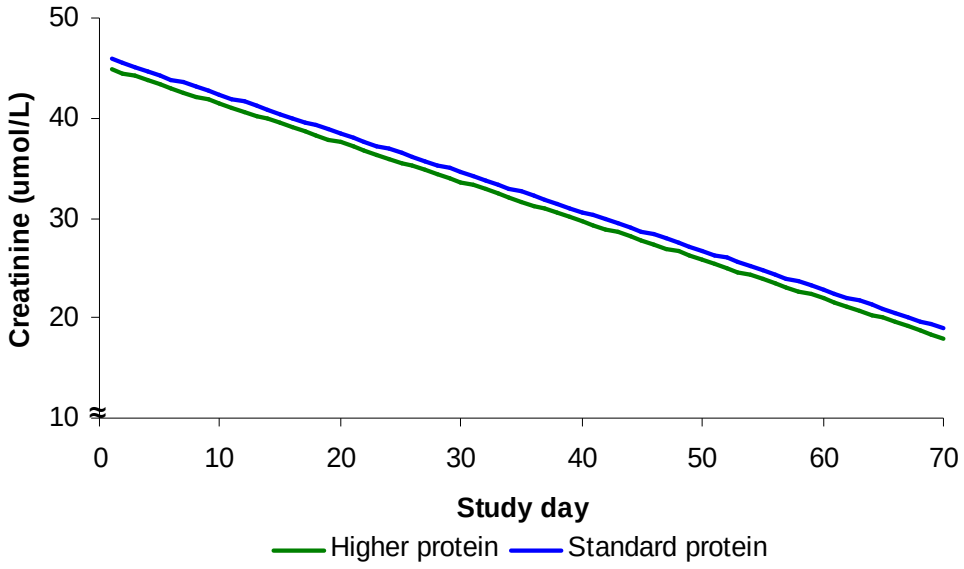


Figure 5-4 Adjusted serum creatinine levels during study

5.3.3 Plasma albumin

Unadjusted plasma albumin levels ranged between 18 and 40 g/L over the course of the study and are shown in **Figure 5-5**. There were 19 infants (on 38 occasions) with hypoalbuminaemia, defined as a serum level below 25 g/L, and these infants were evenly distributed between the groups with 10 and 9 infants in the standard and higher protein groups, respectively.

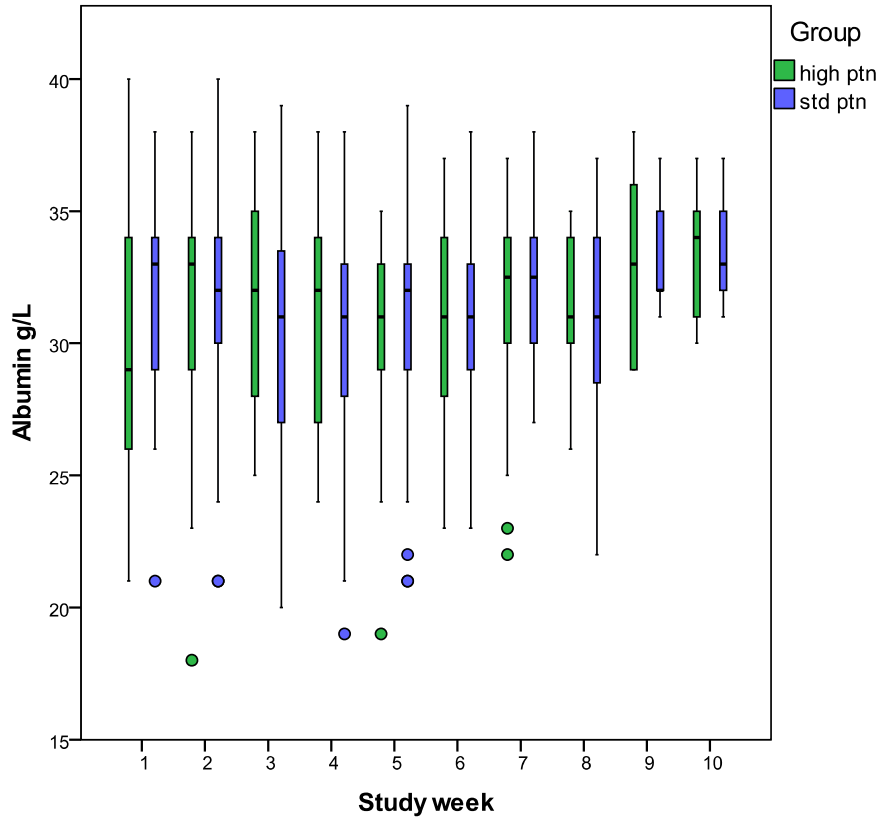


Figure 5-5 Unadjusted weekly albumin levels
Boxes represent interquartile range with median represented by line; whiskers represent 1.5 x interquartile range, outliers (circles) are defined as points > 1.5 box-lengths from the edge of the box.

The equation used for estimating adjusted albumin levels was:

$$Albumin\ (gl/L) = 29.92 + 0.06 * SD - 0.52 * groupA$$

Equation 5-3 Estimated albumin levels, where SD = study day, Group A = 1 if the infant is in the standard protein group and Group A = 0 if the infant is in the higher protein group

Adjusted plasma albumin levels increased significantly over the course of the study, independent of group ($p < 0.0001$) and are shown in **Figure 5-6**. There was no significant difference in plasma albumin levels between the groups ($p = 0.4$).

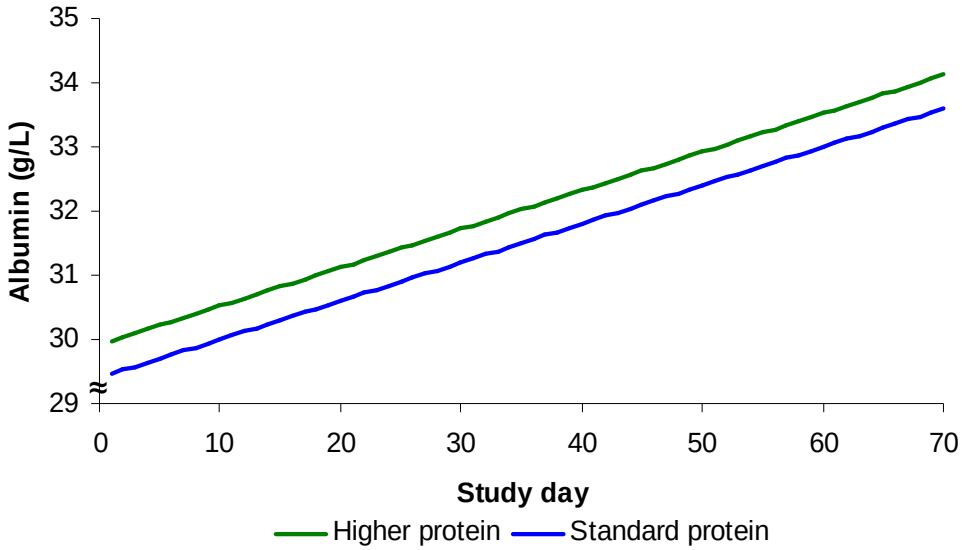


Figure 5-6 Adjusted albumin levels during the study

5.3.4 Blood pH

Unadjusted pH levels are shown in **Figure 5-7** and demonstrate considerable overlap between the treatment groups.

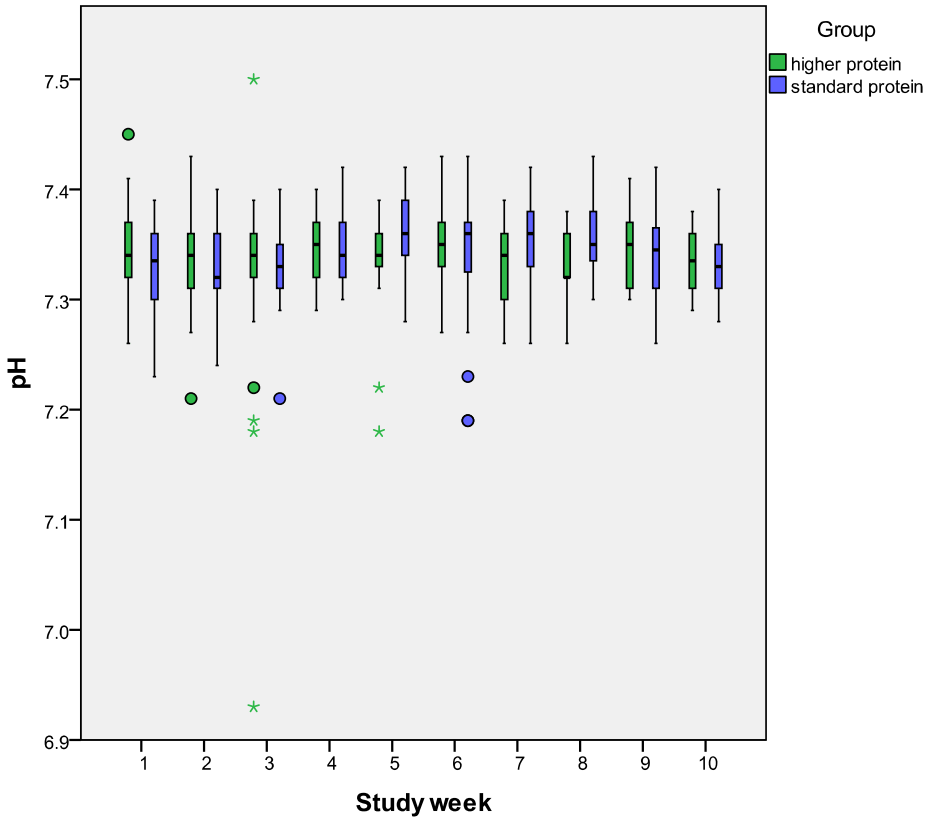


Figure 5-7 Unadjusted pH levels during study
Boxes represent interquartile range with median represented by line; whiskers represent 1.5 x interquartile range, outliers (circles) are defined as points >1.5 box-lengths from the edge of the box. Extreme values (*) are >3 IQR from end of box. 3 extreme values not displayed on graph due to scale: Week 3, 5.9 (Std protein group). Week 6, 6.6 and 6.7 (both higher protein group).

The equation used to estimate pH using our adjusted model was:

$$pH = 7.334 + 0.0003 * SD - 0.0012 * GpA$$

Equation 5-4 Estimated pH levels, where SD = study day, Group A = 1 if the infant is in the standard protein group and Group A = 0 if the infant is in the higher protein group

Adjusted blood pH levels are shown in **Figure 5-8**. Levels increased significantly over time, independent of group (p = 0.002). There was no significant difference in blood pH between treatment groups (p = 0.8)

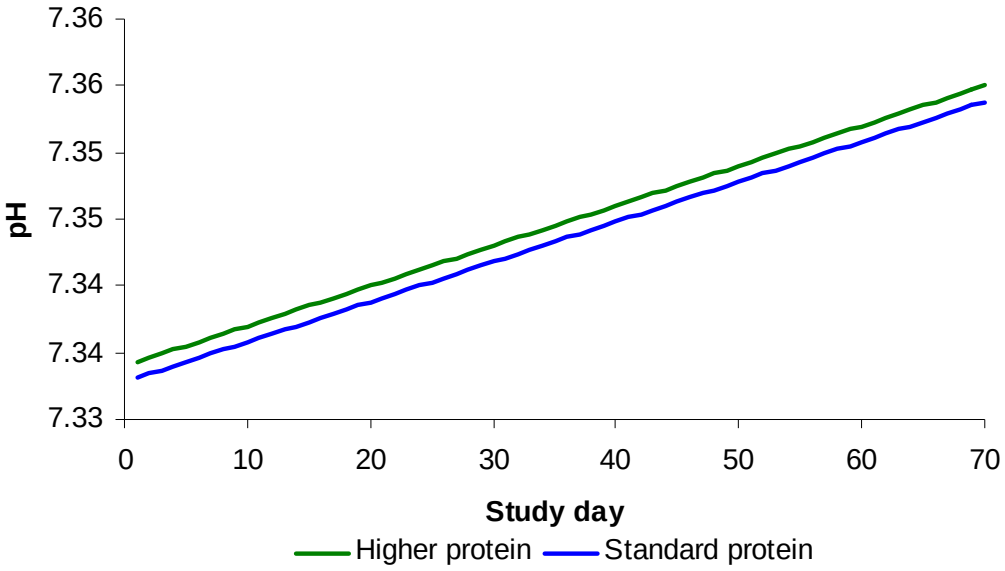


Figure 5-8 Adjusted pH levels during study

5.4 Whole blood amino acids

Whole blood amino acid levels were determined using Tandem Mass Spectrometry by the WCH Neonatal Screening Laboratory, as discussed in **Section 3.4.5**. Levels were compared with 2001—2003 South Australian Neonatal Screening Centre reference values which were based on the average of the metabolic profiles from 12,161 term infants without any diagnosed inborn errors of metabolism. Levels were defined as high if they were above the 99th percentile of the reference values.

5.4.1 Phenylalanine

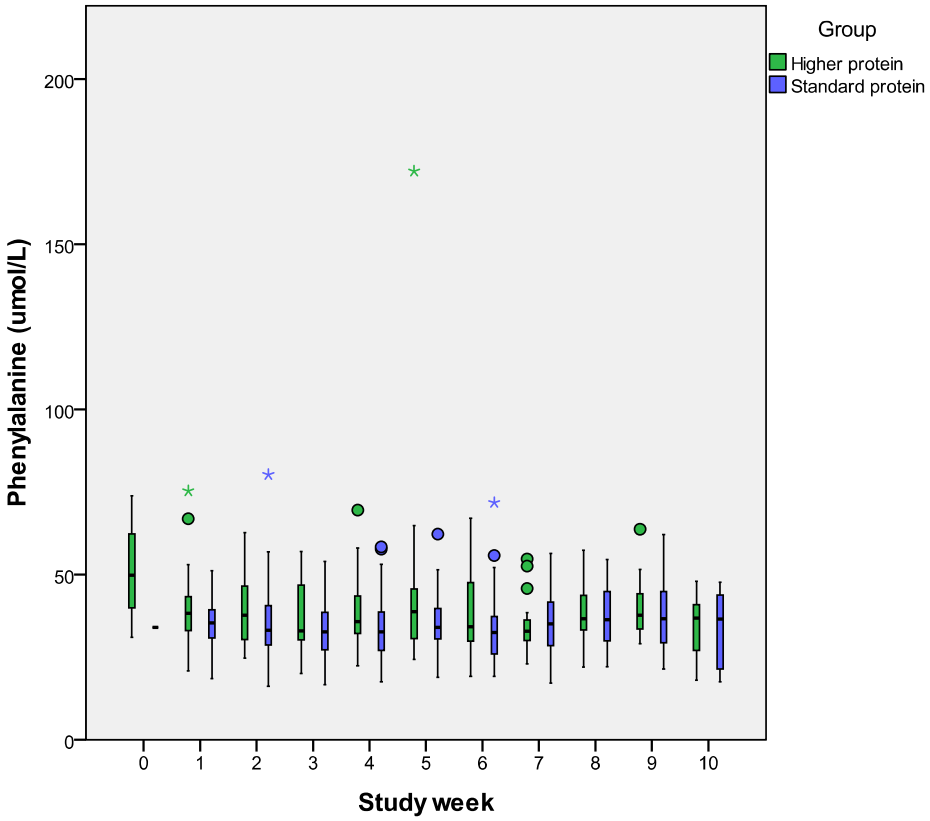


Figure 5-9 Unadjusted phenylalanine levels during the study
Boxes represent interquartile range with median represented by line; whiskers represent 1.5 x interquartile range, outliers (circles) are defined as points >1.5 box-lengths from the edge of the box. Extreme values (*) are >3 IQR from end of box.

Unadjusted phenylalanine levels are shown in **Figure 5-9** and ranged between 16.2 and 172.1 µmol/L. The 99th percentile of the WCH reference data was 76 µmol/L. There were 3 infants who recorded levels above 76 µmol/L. One of these infants, in the standard protein group and fed fully on fortified EBM, recorded a level of 80.3 µmol/L on study day 10, which had dropped to 48.4 µmol/L a week later. Another infant, in the higher protein group but fed fully on preterm formula, recorded a level of 88.1 µmol/L just prior to discharge and this was not followed up. The third infant in the higher protein group recorded the

highest level of 172.1 µmol/L. This infant had an infection at the time of the test, and was on both enteral and parenteral nutrition. Subsequent tests were all within the normal range. All other biochemical results for this infant were normal at the time of this high phenylalanine level. **Table 5-1** compares the least square means of the two groups and demonstrates a significantly higher phenylalanine level in the higher protein group ($p = 0.002$).

Table 5-1. Least square means of phenylalanine by treatment group (µmol/L)

Group	Mean	Std Error
Higher protein	38.77	0.95
Standard protein	34.64	0.93

$p = 0.002$

5.4.2 Tyrosine

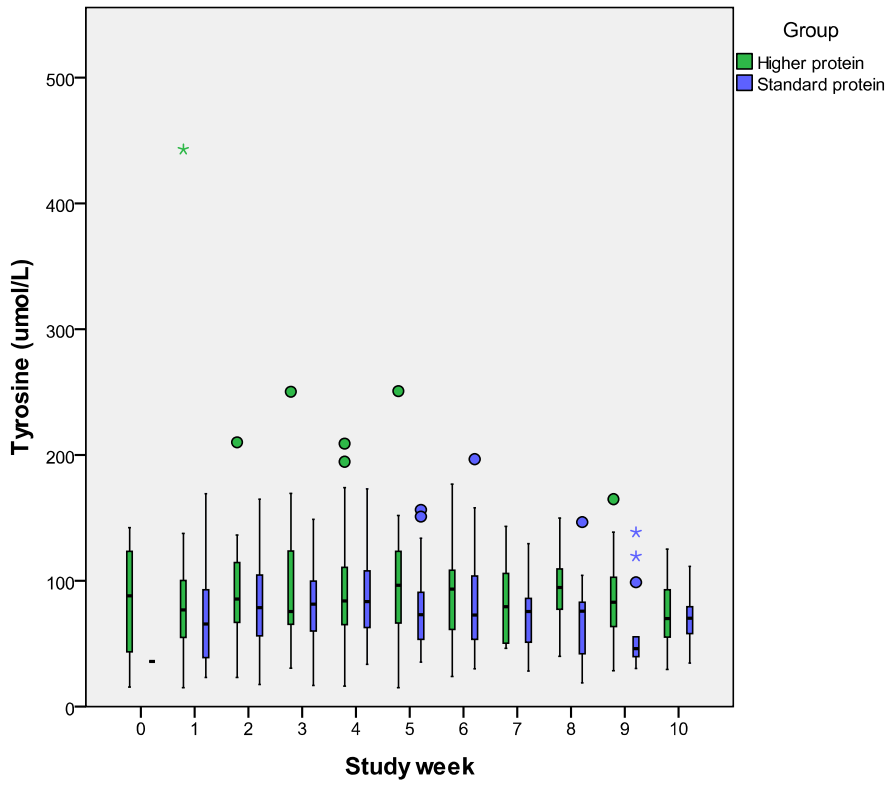


Figure 5-10 Unadjusted tyrosine levels during the study
Boxes represent interquartile range with median represented by line; whiskers represent 1.5 x interquartile range, outliers (circles) are defined as points >1.5 box-lengths from the edge of the box. Extreme values (*) are >3 IQR from end of box.

Unadjusted tyrosine levels in the study ranged between 15 and 442.8 µmol/L (**Figure 5-10)**. The 99th percentile of the WCH reference data was 192 µmol/L. There were 5 infants with levels above this cut off, 1 and 4 in the standard protein and higher protein groups, respectively. Three infants, including the infant in the standard protein group, recorded levels slightly above the cut off (up to 210 µmol/L) and these all returned to normal in subsequent tests. Another 2 infants, both in the higher protein group, recorded 2 levels above the cut off (209 and 250.7 µmol/L in one infant with all other levels within normal, and 442.8 dropping to 250.3 µmol/L the only 2 levels done in the other infant). **Table 5-2**

shows significantly higher adjusted tyrosine levels in the higher protein group (p = 0.04).

Table 5-2. Least square means of tyrosine by treatment group (µmol/L)

Group	Mean	Std Error
Higher protein	92.2	5.2
Standard protein	78.0	3.7

p = 0.04

5.4.3 Branched chain amino acids

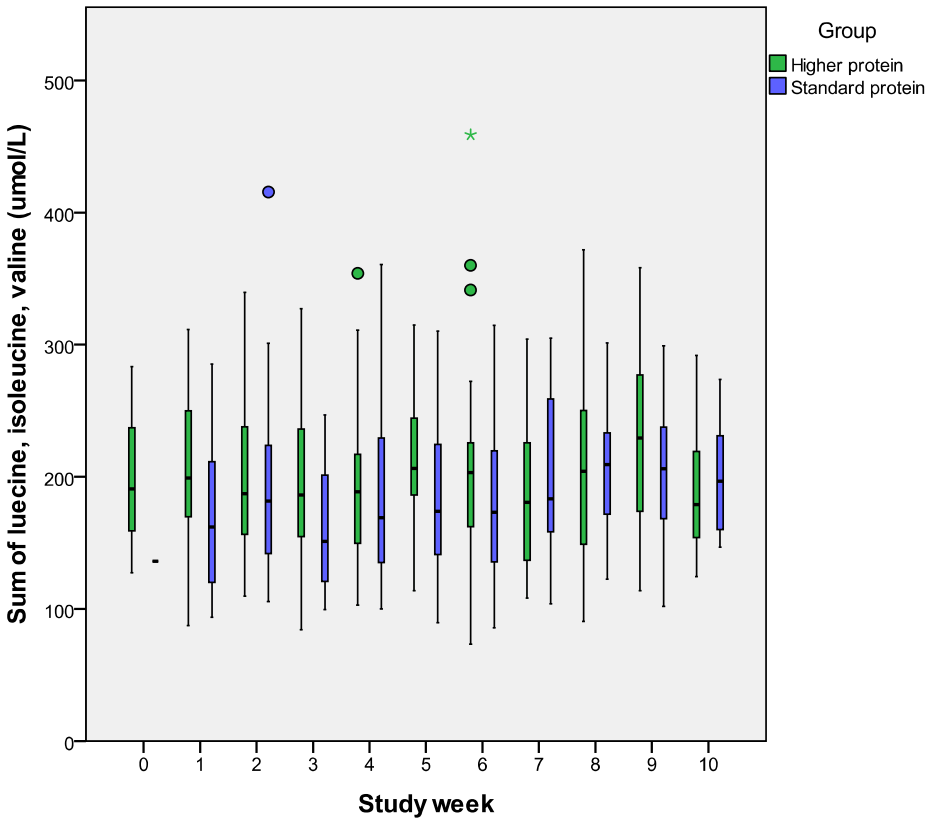


Figure 5-11 Sum of branched chain amino acids (leucine, isoleucine and valine) during the study
Boxes represent interquartile range with median represented by line; whiskers represent 1.5 x interquartile range, outliers (circles) are defined as points >1.5 box-lengths from the edge of the box. Extreme values (*) are >3 IQR from end of box.

The branched chain amino acids (BCAA), leucine, isoleucine and valine, were summed to simplify comparison of the data. The 99th percentile for branched chain amino acids was 346 µmol/L and was calculated by summing the individual percentiles for valine and leucine / isoleucine (174 and 172 µmol/L, respectively). Leucine and isoleucine have the same atomic weight and so are not separated by tandem mass spectrometry. Levels ranged between 73 and 459 µmol/L in the study with 7 infants recording levels above the cut off (**Figure 5-11**). Two of these infants were in the standard protein group and one of these received a mixture of fortified EBM and preterm formula at the time of the test. Of the five infants in the higher protein group, one was fed wholly on preterm formula and the others were fed wholly on fortified EBM. **Table 5-3** shows the least square means of the sum of leucine, isoleucine and valine in the groups and demonstrates significantly higher levels in the higher protein group (p = 0.01).

Table 5-3. Least square manes of branched chain amino acids (µmol/L)

Group	Mean	Std Error
Higher protein	201.6	5.5
Standard protein	181.9	5.8

p = 0.01

5.5 Results Clinical Outcomes

5.5.1 Gastrointestinal outcomes

The number of days that feeds were interrupted, defined as one or more feeds withheld during a 24 hour period, was used as a marker of feeding tolerance. **Table 5-4** indicates there was no difference between groups (p = 0.7). Eight

infants were diagnosed with NEC during the study, 3 (7%) from the higher protein group and 5 (10%) from the standard protein group. A Chi-square test for independence with Fishers Exact Test showed no difference between the groups in the incidence of NEC (p = 0.719). Three infants with NEC required bowel surgery, 2 in the standard protein group and 1 in the higher protein group (**Table 5-4**).

Table 5-4. Gastrointestinal outcomes

	Higher protein (n = 43)	**Standard protein (n = 49)**
No. days feeds were interrupted*, mean (SD)	3 (5)	4 (6)
Infants diagnosed with NEC, n (%)	3 (7)	5 (10)
Infants requiring bowel surgery, n (%)	1 (2)	2 (4)

*Defined as one or more feeds held in a 24 hour period

5.5.2 Respiratory outcomes

Oxygen requirement at 36 weeks PMA was used as an indicator of respiratory function. There were 20 infants (47%) requiring oxygen in the higher protein group and 17 (35%) in the standard protein group. The Relative Risk for requiring oxygen at 36 weeks with higher protein intake, adjusted for sex, GA and sibling clustering, showed no significant difference between the groups (RR 0.96, CI 0.7 – 1.2. p = 0.7).

5.5.3 Retinopathy of prematurity

Most infants were assessed for retinopathy of ROP by an ophthalmologist. Four of the most mature infants (all born at 30 weeks gestation) were not assessed, 3 in the higher protein group and 1 in the standard protein group. There was no difference in the number of infants diagnosed with ROP (p = 1) or the proportion with ROP ≥ grade 3 (p = 0.4). Only one infant in the higher protein group required laser surgery for severe ROP (**Table 5-5**).

Table 5-5. Incidence of retinopathy of prematurity (ROP)

	Higher protein (n = 43)	**Standard protein (n = 49)**
Infants assessed for ROP, *n*	40	48
Infants with ROP diagnosed, *n (%)*	13 (30)	15 (31)
Infants with severe ROP (≥ grade 3), *n (%)*	3 (7)	2 (4)
Infants who required laser surgery, *n (%)*	1 (2)	0

5.5.4 Other clinical outcomes

Table 5-6 shows the incidence of some outcomes that reflect the clinical course of the infants in the two groups. There was no difference in the number of infants requiring surgery (p = 0.87). The most commonly required surgery was Patent Ductus Arteriosis (PDA) ligation or repair of inguinal hernia. The incidence of brain injury was not different between the groups (p = 0.9) and one infant in each group had a severe IVH (defined as ≥grade 3). Sepsis occurred in 6 and 7 infants in the higher and standard protein groups, respectively. Two infants (both in the standard protein group) had two episodes of sepsis. There was no difference between the groups in the incidence of sepsis (p = 0.1) or chorioamnionitis (p =

0.39). Approximately a third of infants required postnatal steroids and this proportion was not different between the groups (p = 1). The mean length of hospital stay was not different between the groups (p = 0.36), nor was the PMA at discharge (p = 0.76).

Table 5-6 Clinical outcomes

Variable	**Higher protein n = 43**	**Standard protein n = 49**
Infants who required surgery, n (%)		
Any surgery	13 (30)	13 (27)
PDA ligation	4 (9)	5 (10)
Inguinal hernia	8 (19)	6 (12)
Infants with brain injury, n (%)		
Any	10 (23)	13 (27)
IVH ≥ grade 3	1 (2)	1 (2)
Infants with sepsis, n (%)	6 (14)	7 (14)
Episodes of sepsis	6 (14)	9 (18)
Chorioamnionitis	15 (35)	11 (22)
Infants requiring postnatal steroids, n (%)	14 (33)	17 (35)
Length of stay, mean (SD), days	77 (21)	73 (16)
Postmenstrual age at discharge, mean (SD), weeks	38.2 (1.5)	38.4(1.4)

5.6 Discussion

5.6.1 Biochemical data

Urea is the biochemical product of protein degradation and reflects dietary protein intake, hydration status and renal function. Serum urea concentration was used in this study as an indicator of protein nutritional status as it is proportional to dietary

protein intake (153-155), has a shorter half life than other markers of protein metabolism such as albumin, and is readily available as it is monitored as part of routine clinical care. In this trial, both urea and creatinine decreased over time independent of the infant's group allocation and it is possible that this relates to improving glomerular filtration rates as the infants matured. The significantly higher urea levels found in the higher protein group support the success of the intervention and indicate higher protein intakes in this group. This is consistent with other studies varying the protein concentration, both in fortified EBM (127, 128, 151) and formula studies (11). Seven infants in this trial developed transient uraemia (defined as >8 mmol/L) and this is in contrast with other trials where levels were all within the accepted range (11, 150, 151). However, the definition of uraemia varied in these studies, with Porcelli *et al* using 10mmol/L as the upper limit, Cooke *et al*, using 7 mmol/L and Reis *et al* not defining the normal range. In addition these studies enrolled more mature infants with Porcelli *et al* (151) and Cooke *et al* (11) enrolling infants born ≤32 weeks gestation and Reis *et al* (150) enrolling infants born ≤33 weeks gestation. Of the seven infants with transient uraemia in my trial, five of these were born <28 weeks gestation. All high levels were recorded within the first 10 days of the study and subsequently resolved without intervention. Many more infants recorded low urea levels (≤1 mmol/L), and the majority of these were in the standard protein group (11 out of 15), possibly indicating poor protein status, although both hydration status and the maturity of the urea cycle of very preterm infants can complicate interpretation of these results (192, 193).

Plasma albumin levels increased over the course of the study, independent of group. It is known that plasma albumin levels increase with gestation and this is probably due to increased synthesis of albumin by the liver as the infant matures (194, 195). Although the level appeared to be higher in the higher protein group there was no statistically significant difference. Albumin is not a good indicator of short term changes in protein nutritional status because of its long half life and ability to be affected by inflammation and hydration status.

Blood pH levels increased over the study, towards normal, independent of group. A low blood pH can be the result of either respiratory or metabolic acidosis, both of which are relatively common in very preterm infants. Infants in the higher protein group would have incurred a higher acidic load to excrete because of the extra protein. However, there was no evidence of increased acidosis in this group.

5.6.2 Whole blood amino acid levels

Phenylalanine, tyrosine and the sum of the BCAA were all significantly higher in the higher protein group. Other recent HMF trials have not studied amino acid levels so it is not known if this is an unusual finding. Moro *et al* (146), in 1991, compared plasma amino acid levels in infants fed human milk fortified with either human milk protein or a bovine fortifier and found no differences in levels between the groups. However, protein intakes in this study were 3.3 to 3.6 g/kg/day (146), whereas infants in my trial had intakes 3.6 to 4.5 g/kg/day. Moro *et al* (128) in a later study comparing fortifier added in a fixed rate versus a rate adjusted by the infants' BUN level, found higher plasma levels of amino acids in the adjusted group. These levels were similar or higher to those in my trial,

(phenylalanine 38.0 vs 38.7; tyrosine 102.0 vs 92.2; BCAA 268.0 vs 201.6 µmol/L for levels in my trial and Moro respectively). However, Moro et al measured plasma levels whereas my trial used whole blood levels of amino acids so the two results may not be directly comparable. Moro did not report instances of amino acid levels about the normal range. In my trial, 12 infants recorded amino acid levels above the 99th percentile of our reference data on a total of 17 occasions. Four of these occasions occurred when the infant was fed either preterm formula (2 occasions) or a mixture of formula and fortified EBM (2 occasions). There was one occurrence of a very high phenylalanine level in an extremely sick infant and this resolved spontaneously as did high levels in other infants. The fact that transient high levels occurred even when infants are fed preterm formula raises the possibility that this happens routinely at times without our knowledge, because it is not monitored. Blood amino acid levels also vary depending on the timing of sampling in relation to infant feeding. Because of the workload in the neonatal unit, it was not possible to standardise blood collection to a fasting specimen only. The clinical significance of transient high amino acid levels is not clear.

5.6.3 Clinical outcomes

The Australian and New Zealand Neonatal Network (ANZNN) reports annual data on preterm births from all level III neonatal intensive care units in Australia and New Zealand (196). From this report, the 2006 (most recent data available) incidence of NEC was 1.9% in all infants and 13.3% in infants born <28 weeks gestation (196). For NEC, data are not available for infants born ≤30 weeks gestation for direct comparison with the infants in the current trial. However, in

my trial, the majority of infants who developed NEC were born <28 weeks gestation (6 out of 8). The incidence in this subgroup of infants was 13.9%, comparable with the Australian and New Zealand data (13.3%). The overall incidence of NEC in my trial was 8.7% and there was no difference in the incidence between groups. Similarly Reis *et al* (150) did not show a difference between groups in the incidence of NEC. However, most studies are not powered to detect these small differences and some trials have used NEC as an exclusion criterion (128). There are some concerns that the fortification of milk increases the osmolality (190) and reduces anti-infective properties (197) of breast milk and these factors may interact to increase the incidence of NEC. Lucas *et al* (72) found a higher rate of NEC in preterm infants fed fortified (8 out of 137, 5.8%) compared to unfortified EBM (3 out of 138, 2.2%). Despite the fact that this did not reach statistical significance, it has raised some concerns about fortification. Because the incidence of NEC is relatively low, much larger trials are required to detect a significant difference. There were no other differences in feeding tolerance between the groups in my trial.

Chronic lung disease (CLD) is defined as those infants who continue to require respiratory support at 36 weeks PMA (196). The rate of CLD in my trial was 47% and 35% in higher and standard protein groups, respectively. This is higher than available national average data which is 13.7% for all infants born <32 weeks and 38.9% for those infants born <28 weeks (196) and the reason for this is not clear. CLD is known to be inversely proportional to GA (196) and my trial included six infants born <24 weeks gestation which may have skewed the figures. However, there were no significant differences between groups in my trial.

Rates of ROP in my trial (30% for the entire cohort) were comparable with the national average for all infants of approximately 27%. There was no difference between groups in my trial. Similarly, rates of severe ROP (defined as stage 3 or 4) in my trial were 5%, similar to the national average of 4% (196).

Other clinical outcomes were recorded and compared with national data to monitor any differences between groups and to determine the representability of this cohort to the general neonatal population. There were very few exclusion criteria in my trial and even sick and very immature infants were enrolled. The incidence of brain injury in my trial was 25%, comparable with national data with an incidence of 24.4%. Similarly, the incidence of IVH of ≥grade 3 was 2% in my trial, relative to the national average of 3.9% (196). Length of stay and PMA at discharge did not differ between the groups. This is despite the fact that the higher protein group contained 5 of the least mature infants who would be expected to have longer lengths of stay. It is possible that the extra protein attenuated this effect. Other HMF studies have not reported length of stay or age at discharge.

5.7 Summary

This trial has shown that higher protein fortifier has improved the protein nutritional status of infants as evidenced by higher serum urea levels together with a lower incidence of urea levels ≤1 mmol/L. However, a small proportion of infants developed transient uraemia. Similarly, higher blood amino acid levels are indicative of an increase in dietary protein, yet a small number of infants recorded

transient levels above normal. The fact that high levels of urea and amino acids all resolved spontaneously without intervention indicates that this level of dietary protein is safe, but requires monitoring. In particular, this trial is generalisable to current neonatal populations because I included sick and very immature infants, who may be more metabolically vulnerable than the healthy preterm infant population sometimes included in other trials.

Other clinical data collected provides an indication of the general morbidity and indicate that the infants included in this study are comparable with national data.

Chapter 6 General Discussion

6.1 Introduction

From a nutritional view point, premature birth can be likened to a physiological insult as these infants must rapidly adapt from an intra- to an extra-uterine environment. While *in-utero*, predigested nutrients are available 'on tap' via the placenta at a rate that meets their high demand for tissue accretion. Extra-uterine conditions require intact nutrients to be delivered via an immature digestive tract while equally immature body systems must cope with their digestion and metabolism. Frequently, the nutritional management of the infant is not initially the greatest priority, as other body systems, such as respiratory, need to be stabilised. However, evidence is mounting that this early time is critical in their development and can influence long term outcomes such as neurodevelopment and lifestyle disease (**Section 1.2**).

It is well recognized that breast milk provides unique advantages to infants, but that it is inadequate on its own for preterm infants and hence fortification of EBM is an established practice (**Section 1.2.2.1**). Despite this, there is overwhelming evidence of unsatisfactory growth in infants fed breast milk and discussion in the literature regarding the inadequacies of current EBM fortification regimes and commercially available fortifiers (**Section 1.2.4**). One of the two main issues with current fortification regimes is their failure to take into account the fall in the breast milk protein concentration that takes place in the first few weeks after birth. This thesis has attempted to address this issue by validating a milk analyser to accurately and quickly measure the protein and fat content of EBM (**Chapter 2**).

The second issue relates to the protein content of commercial fortifiers. Given that very preterm infants are such a vulnerable and diverse group of infants, it is understandable that clinicians and fortifier manufacturers have a conservative approach to adding protein, in an attempt to avoid the metabolic consequences of protein overload. Some investigators have dealt with this problem by using an individualised regime of fortification, based on either the protein content of the EBM or the biochemical response of the infant (**Section 1.2.6**). Results of trials implementing the individualised regimes have confirmed the fact that most infants require more protein most of the time and that very few infants require less protein and then only some of the time (127, 128). While this tailor-made solution seems ideal, it is both time and resource consuming. It may be more worthwhile to have a general approach to supplying more protein and then focus individualised attention to those infants who need it, as indicated by growth and biochemical monitoring.

My hypothesis was that preterm infants fed EBM fortified with a higher level of protein (1.4 g/100mL) will have improved growth (length, weight and head circumference gains), higher biochemical markers of protein metabolism (SUN and albumin) but no increase in biochemical disturbances or adverse events when compared with infants fed EBM fortified at standard levels (1.0 g/100mL).

6.2 Key findings

The growth outcomes of this trial are discussed in **Chapter 4.** Length gain was chosen as the primary outcome as it is a better indicator than other growth parameters of lean tissue accretion, which we would expect to respond to an

increase in dietary protein intake at a constant energy intake. There was a small increase in mean weekly length gain in the higher protein group (1.15 cm/wk, 95% CI 1.1 – 1.19) when compared with the standard protein group (1.09 cm/wk, 95% CI 1.05 – 1.13) but this did not reach statistical significance ($p = 0.08$). However, this small weekly divergence was enough to translate into a difference in the number of infants classified as SGA for length at discharge (49% compared with 63% in the higher and standard protein groups respectively, $p = 0.047$). Nevertheless, these SGA figures highlight that there is still considerable room for improvement in the growth of preterm infants and that the higher protein did appear to improve the situation. Future trials could be directed toward comparisons of fortifiers with even higher protein concentrations as we may still not be meeting infants' requirements. In the exploratory analyses, length gain was consistently greater in the higher protein group but this only reached statistical significance in boys and infants born 28 – 30 weeks gestation. Small sample size makes interpretation of these results difficult. Larger trials, designed with an adequate sample size to detect differences in subgroups of interest such as sex and gestational age, are required.

Chapter 5 reports the biochemical and clinical events data. The higher protein group had higher levels of SUN and amino acids but not albumin and creatinine. While there were some instances of levels of both SUN and amino acids above the normal range which occurred more frequently in the higher protein group, these all resolved without intervention. Perhaps of greater concern was the number of infants with low levels of protein metabolites, indicating possible protein malnutrition. There were no differences in other clinical outcomes.

Overall, while the findings do not support the primary hypothesis of improved length gain using a higher protein fortifier, at the probability level of <0.05, they do suggest an improvement in growth and thus protection from a classification of SGA for length at discharge. The secondary hypothesis of higher protein metabolites with no increase in adverse effects has been supported.

6.3 Study strengths and limitations

This study is unique because the objective was to test two different protein levels of fortifier at the same energy level by varying carbohydrate. Other nutrients were also balanced. Previous fortifier comparison trials have evaluated the effect of two fortifiers of quite different composition (137, 142, 143, 148-152) or have used the same fortifier in different amounts, so that all nutrients were in greater supply, not just protein (127, 128, 130). My study has also used a higher protein concentration than any other study.

This study also addresses a number of design and implementation flaws in previous trials. The study population was specified to allow broad generalisability to very preterm infants. Sick and very small infants were included, as was mixed feeding as this is typical of modern neonatal units. Randomisation and allocation concealment were done according to the CONSORT statement. The fortifiers used were made specifically for the trial and were identical in packaging, appearance and rate of mixing, making it possible to completely blind the trial to everyone involved in it. Other studies used fortifiers requiring either a different rate of mixing (149, 151), different vitamin fortification regimes (148) or

compared a liquid with a powdered fortifier (149); all making blinding impossible. I was concerned that the small difference in protein between the fortifiers may not be sufficient to differentiate between the groups, given the considerable variation in the protein content of the EBM, and the dilution effect of the formula used. However, I was able to show a difference in dietary protein intake over the first 4 weeks, between the groups of the expected magnitude, indicating the success of the intervention (**Section 4.4.2**). Considerable effort was invested in measuring length gain; the primary outcome. Appropriate equipment was used; assessors were limited to 4 across 2 sites; all were trained using a protocol which specified tolerance of the 2 measures taken and the inter-rater variability was low. All analyses were done according to 'intention to treat' principles.

The limitations of this trial must be considered when interpreting the results. There was a significant imbalance of multiple births in the groups which I did not anticipate. We attempted to counteract for this by using a statistical model that included sibling clustering. However, this may not have been adequate. In future studies, it is essential that stratification for multiple births occurs.

Additionally, while I stratified for sex and GA, the sample size was not powered to undertake subgroup analyses, limiting the interpretation of these secondary analyses.

Missing data and necessary assumptions about the macronutrient content of the EBM also limited the analyses. It was not possible to collect an EBM sample weekly as I had anticipated due to either low supply of the mother or logistical

reasons relating to ward routines. I opted to collect an EBM sample weekly and extrapolate the protein and fat content of this sample to the week. However, this may have introduced some inaccuracies as the protein content falls quite quickly, particularly in the early weeks after birth. A more accurate result could have been attained by pooling 1 mL of milk collected daily and analysed weekly. However, I did not have sufficient time and resources for daily collections. These decisions limited my ability to accurately report dietary intakes. However, I was able to accurately report the additional protein added from the fortifier.

The heterogeneity in the nutrition intervention also increased as the study progressed, both within and between treatment groups, due to the introduction in the number of direct breastfeeds and the increased use of infant formula. However, these were both post-randomisation variables, and as such were not able to be predicted or controlled.

6.4 Reflections on this randomised controlled trial

Sample size calculations for my trial were based on Arslanoglu *et al's* study of adjusted fortification which resulted in a protein difference of approximately 0.4 g/100mL, similar to the difference between fortifiers used in my trial (127). It was therefore surprising to find a much smaller and non-significant effect on linear growth with this increase in protein in my trial and interesting to postulate why this may be so. **Figure 4-14** compares the difference in length gain found with changing protein concentrations in other studies where energy has been kept constant. In these and Arslanoglu's study, the protein intakes are considerably lower than in my trial and there is a greater effect on growth. It is possible that at

lower protein intakes, requirements are not being met for the majority of infants and hence an increase in protein may better meet their requirements and have a more pronounced effect on growth. The protein content of the control fortifier used in my study added 1 g protein/100 mL EBM and is comparable to the higher protein intakes in these other trials. The control fortifier used in Arslanoglu's study added 0.8 g protein/100 mL. One explanation for the lack of a statistically significant effect seen in my study is that as protein intakes approach requirements the effect on growth may be less dramatic. Either a larger sample size with enough power to detect small differences or a greater differential in protein intake between the fortifiers is necessary to answer these questions satisfactorily. Another likely possibility is that the imbalance of twins and the very conservative statistical modelling in my study has affected the results.

6.5 Implications for practice

While individualised fortification regimes resolve the problems of both protein deficit and excess, they are time and resource consuming to implement and this trial suggests that fears about protein overload may be overstated. Given that infants generally need more protein rather than less, and can tolerate it at the levels used in my trial, it would seem practical to increase the protein content of commercially available fortifiers. Monitoring of these vulnerable infants is still of paramount importance, and SUN, in conjunction with other clinical indicators, could be used to identify infants who may benefit from an individualised regime.

Another approach to better meeting nutrient requirements for preterm infants is to increase feed rates, as they do at Auckland City Hospital, Auckland, New Zealand (34). The target feeding rate used is 180 mL/kg/day and, in an audit of their

feeding practices, energy intakes were above requirements and protein intakes were at the lower end of the range for ELBW infants (34). Weekly mean weight gain was adequate however by 36 weeks approximately a third of infants were classified as SGA for weight. If protein, rather than energy, is the limiting factor for weight gain, increasing feed rates may result in an oversupply of some nutrients such as energy, while still failing to provide adequate protein.
Many neonatalogists adopt a more conservative enteral feeding policy because of concerns about the rate of NEC and other problems with fluid overload. Despite the fact that Auckland City Hospital have low rates of NEC (34), feeding rates as high as 180 mL/kg are not widely used.

The RCT reported in this thesis suggests that it is safe for this population of infants to receive higher doses of protein through fortified EBM. This extra protein may translate into better growth but larger studies are required to substantiate these findings. Growth data from this and other trials suggest that there is still substantial room for improvement in the growth of very preterm infants and therefore, nutritional management of these infants. In my trial, the higher protein protected against a classification of SGA for length at discharge. The Number Needed to Treat (NTT) to prevent SGA, calculated as the inverse of the absolute risk reduction, was 5 (95% CI 2.7 – 117) i.e. 5 infants would need to be given higher protein fortifier in order to prevent 1 infant from being classified as SGA for length at discharge. The confidence intervals for this NNT are wide, implying poor precision, possibly due to the small sample size. The investigation of SGA was a *post hoc* analysis and this study was not powered to detect these changes.

6.6 Future directions

The higher protein fortifier used in my trial achieved median dietary protein intakes of between 4 and 4.5 g/kg/day during the first 4 weeks of the study (**Table 4-7**) which meets theoretical protein requirements of 3.5 to 4.5 g/kg/day (12, 27). However, after this time the number of direct breast feeds increased, thereby diluting the effect of the higher protein fortifier. Therefore protein intakes of preterm infants fed human milk can be predicted to be lower than infants fed preterm formula because, as they progress to direct sucking feeds, they receive unfortified milk whereas formula fed infants continue to receive the same formula. This effect is intensified by the fact that the protein content of the mother's milk reduces over time, approaching levels of mothers who have delivered term infants by about 4 weeks postpartum. By the time the infant is mature enough for direct breast feeds, the protein content of the milk is significantly lower than preterm formula. A review of the nutrient requirements for preterm infant formula (12) has suggested a protein energy ratio of 2.5 - 3.6 g/100 Kcal. The higher protein fortifier in my trial provided 3.2 g protein/100 Kcal, calculated using the mean protein content of the EBM from my study (1.27 g/100mL). This is within the recommended range and, considering that this is diluted even further when these infants initiate direct sucking feeds, suggests that an even higher level of protein could be used in fortifier. Indeed, Cooke *et al* (11) used a preterm formula with 3.6 versus 3.0 g protein/100 Kcal in a metabolic balance study and found better protein accretion and weight gain with no metabolic disturbances with the higher protein formula. In addition, Arslanoglu *et al*, in their adjusted fortification study (127) reached a mean level of adjustment of +2.3 in week 3 which equates to

approximately an extra 0.7 g protein/100 mL. Both these studies suggest the possibility that protein levels in fortifier could be increased even further than the upper level used in my trial. However, as fortifier is added to EBM with a highly variable protein content, dietary intake modelling would be necessary to establish potential upper limits of intakes.

Finally, while growth is an important outcome for nutritional intervention studies, it would be also be useful to monitor the composition of the weight gain (i.e. the proportion of lean and fat mass accretion). This could then be related to long term health outcomes such as the incidence of Metabolic Syndrome in later life. Neuro-development is another important long term outcome for this group that may be related to growth and early diet. Large sample sizes are needed to detect these differences.

6.7 Conclusion

In conclusion, increasing the protein content of commercially available HMFs seems to be a promising strategy for safely improving the growth of very preterm infants. Very few studies have examined protein at levels similar to or higher than the concentrations used in my trial and this is a potential area for future research. Preterm infants are a heterogeneous group and it is possible that particular subgroups may be more responsive to higher protein. Large studies with sufficient sample size to detect differences in subgroups of interest (sex and GA) would help to clarify any potential target groups. Long term follow up is necessary to evaluate both the short and long term effects of increasing the protein content of fortifiers. Important short term outcomes include growth, body

composition, biochemical and clinical indicators. Long term outcomes of interest are growth and body composition in later life, the incidence of lifestyle diseases and neuro-development.

References

1. Tracy SK, Tracy MB, Dean J, Laws P, Sullivan E. Spontaneous preterm birth of liveborn infants in women at low risk in Australia over 10 years: a population-based study. BJOG: An International Journal of Obstetrics & Gynaecology 2007;114(6):731-5.
2. Langhoff-Roos J, Kesmodel U, Jacobsson B, Rasmussen S, Vogel I. Spontaneous preterm delivery in primiparous women at low risk in Denmark: population based study. BMJ 2006;332(7547):937-9.
3. Martin J, Hamilton B, Sutton P, Ventura S, Menacker F, Kirmeyer S, Munson M. Births: Final Data for 2005. National Vital Statistics Reports 2007;56. Internet: http://www.cdc.gov/nchs/data/nvsr/nvsr56/nvsr56_06.pdf accessed 6/01/2009).
4. Laws P, Sullivan E. Australia's mothers and babies 2007. Perinatal statistics series no. 23. Sydney, 2009.
5. Aylward G, Aylward GP. Cognitive function in preterm infants: no simple answers. JAMA 2003;289(6):752-3.
6. Berhrman R, Butler A. US Commitee on Understanding Premature Birth and Assuring Healthy Outcomes. Preterm birth: causes, consequences, and prevention. 2006. Internet: http://newton.nap.edu/catalog/11622.html#toc accessed 10/10/2008).
7. Mangham L, Petrou S, Doyle L, Draper ES, Marlow N. The cost of preterm birth throughout childhood in England and Wales. Pediatrics 2009;123(2).
8. Lucas A, Cole T. Breast milk and neonatal necrotising enterocolitis. Lancet 1990;336(8730):1519-23.
9. Wilson DC, Cairns P, Halliday HL, Reid M, McClure G, Dodge JA. Randomised controlled trial of an aggressive nutritional regimen in sick very low birthweight infants. Archives of Disease in Childhood Fetal & Neonatal Edition 1997;77(1).
10. Thureen P, Hay WW J. Intravenous nutrition and postnatal growth of the micropremie. Clinics in Perinatology 2000;27(1):197-219.
11. Cooke R, Embleton N, Rigo J, Carrie A, Haschke F, Ziegler E. High protein pre-term infant formula: effect on nutrient balance, metabolic status and growth. Pediatric Research 2006;59(2):265-70.
12. Klein C. Nutrient requirements for preterm infant formulas. Journal of Nutrition 2002;132(6 Suppl 1):1395S-577S.
13. McCance R. Food, growth, and time. Lancet 1962;2(7257):621-6.
14. Susser M, Stein Z. Timing in prenatal nutrition: a reprise of the Dutch Famine Study. Nutrition Reviews 1994;52(3):84-94.
15. Law C, Barker D, Osmond C, Fall C, Simmonds S. Early growth and abdominal fatness in adult life. Journal of Epidemiology & Community Health 1992;46(3):184-6.
16. Barker D, Gluckman P, Godfrey K, Harding J, Owens J, Robinson J. Fetal nutrition and cardiovascular disease in adult life. Lancet 1993;341(8850):938-41.
17. Barker D, Osmond C, Simmonds S, Wield G. The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life. BMJ 1993;306(6875):422-6.

18. Barker D, Eriksson J, Forsen T, Osmond C, Barker D. Fetal origins of adult disease: strength of effects and biological basis. International Journal of Epidemiology 2002;31(6):1235-9.
19. Monteiro P, Victora C, Monteiro P. Rapid growth in infancy and childhood and obesity in later life--a systematic review. Obesity Reviews 2005;6(2):143-54.
20. Lucas A, Gore S, Cole T, Bamford M, Dossetor J, Barr I, Dicarlo L, Cork S, Lucas P. Multicentre trial on feeding low birthweight infants: effects of diet on early growth. Archives of Disease in Childhood 1984;59(8):722-30.
21. Lucas A, Morely R, Cole T, Gore S, Lucas P, Crowle P, Pearse R, Boon A, Powell R. Early diet in preterm babies and developmental status at 18 months. Lancet 1990;335(8704):1477-81.
22. Lucas A, Morley R, Cole T. Randomised trial of early diet in preterm babies and later intelligence quotient. BMJ 1998;317(7171):1481-7.
23. Ziegler EE. Nutrient requirements of premature infants. Nestle Nutrition Workshop Series Paediatric Programme 2007;59:161-72; discussion 72-6.
24. Heird W, Kashyap S. Protein and energy requirements of low birth weight infants. Acta Paediatrica Scandinavica - Supplement 1989;351:13-23.
25. Embleton N, Pang N, Cooke R. Postnatal malnutrition and growth retardation: an inevitable consequence of current recommendations in preterm infants? Pediatrics 2001;107(2):270-3.
26. Embleton ND. Optimal protein and energy intakes in preterm infants. Early Human Development 2007;83(12):831-7.
27. Rigo J. Protein, amino acid and other nitrogen compounds. In: Tsang R, Uauy R, Koletzko B, Zlotkin S, eds. Nutrition of the Preterm Infant. Cincinnati Digital Education Publishing, Inc 2005:81 - 96.
28. American Academy of Pediatrics. Nutritional needs of low-birth-weight infants. Pediatrics 1985;75(5):976-86.
29. Cooke R, Rawlings D, McCormick K, Griffin I, Faulkner K, Wells J, Smith J, Robinson S. Body composition of preterm infants during infancy. Archives of Disease in Childhood Fetal & Neonatal Edition 1999;80(3):F188-91.
30. Ernst K, Radmacher P, Rafail S, Adamkin D. Postnatal malnutrition of extremely low birth-weight infants with catch-up growth postdischarge. Journal of Perinatology 2003;23(6):477-82.
31. Ehrenkranz R, Younes N, Lemons J, Fanaroff A, Donovan E, Wright L, Katsikiotis V, Tyson J, Oh W, Shankaran S, Bauer C, Korones S, Stoll B, Stevenson K, Papile L. Longitudinal growth of hospitalized very low birth weight infants. Pediatrics 1999;104(2 Pt 1):280-9.
32. Vohr BR, McKinley LT. The challenge pays off: early enhanced nutritional intake for VLBW small-for-gestation neonates improves long-term outcome. Journal of Pediatrics 2003;142(5):459-61.
33. Collins CT, Gibson RA, Miller J, McPhee AJ, Willson K, Smithers LG, Makrides M. Carbohydrate intake is the main determinant of growth in infants born <33 weeks' gestation when protein intake is adequate. Nutrition 2008;24(5):451-7.
34. Cormack BE, Bloomfield FH. Audit of feeding practices in babies<1200 g or 30 weeks gestation during the first month of life. Journal of Paediatrics & Child Health 2006;42(7-8):458-63.

35. Hay WJ, Lucas A, Heird W, Ziegler E, Levin E, Grave G, Catz C, Yaffe S. Workshop summary: nutrition of the extremely low birth weight infant. Pediatrics 1999;104(6):1360-8.
36. Thureen PJ. Early aggressive nutrition in the neonate. Pediatrics in Review 1999;20(9):e45-55.
37. Berry M, Abrahamowicz M, Usher R. Factors associated with growth of extremely premature infants during initial hospitalization. Pediatrics 1997;100(4):640-6.
38. Pauls J, Bauer K, Versmold H. Postnatal body weight curves for infants below 1000 g birth weight receiving early enteral and parenteral nutrition. European Journal of Pediatrics 1998;157(5):416-21.
39. Smith SL, Kirchhoff KT, Chan GM, Squire SJ. Patterns of postnatal weight changes in infants with very low and extremely low birth weights. Heart & Lung 1994;23(6):439-45.
40. Embleton NE, Pang N, Cooke RJ. Postnatal malnutrition and growth retardation: an inevitable consequence of current recommendations in preterm infants? Pediatrics 2001;107(2):270-3.
41. Cooke R, Ainsworth S, Fenton A. Postnatal growth retardation: a universal problem in preterm infants. Archives of Disease in Childhood Fetal & Neonatal Edition 2004;89(5):F428-30.
42. Steward DK, Pridham KF. Growth patterns of extremely low-birth-weight hospitalized preterm infants. JOGNN - Journal of Obstetric, Gynecologic, & Neonatal Nursing 2002;31(1):57-65.
43. Saigal S, Hoult LA, Streiner DL, Stoskopf BL, Rosenbaum PL. School difficulties at adolescence in a regional cohort of children who were extremely low birth weight. Pediatrics 2000;105(2):325-31.
44. Hack M, Taylor H, Klein N, Eiben R, Schatschneider C, Mercuri-Minich N. School-age outcomes in children with birth weights under 750 g. New England Journal of Medicine 1994;331(12):753-9.
45. Hack M, Breslau N, Weissman B, Aram D, Klein N, E B. Effect of very low birth weight and subnormal head size on cognitive abilities at school age. New England Journal of Medicine 1991;325(4):231-7.
46. Botting N, Powls A, Cooke R, Marlow N. Attention deficit hyperactivity disorders and other psychiatric outcomes in very low birthweight children at 12 years. Journal of Child Psychology & Psychiatry & Allied Disciplines 1997;38(8):931-41.
47. Hack M, Flannery D, Schluchter M, Cartar L, Borawski E, Klein N. Outcomes in young adulthood for very-low-birth-weight infants. New England Journal of Medicine 2002;346(3):149-57.
48. Brandt I, Sticker E, Lentze M. Catch-up growth of head circumference of very low birth weight, small for gestational age preterm infants and mental development to adulthood. Journal of Pediatrics 2003;142(5):463-8.
49. Scott KE, Usher R. Fetal malnutrition: its incidence, causes, and effects. American Journal of Obstetrics & Gynecology 1966;94(7):951-63.
50. Hack M, Merkatz I, Gordon D, Jones P, Fanaroff A. The prognostic significance of postnatal growth in very low--birth weight infants. American Journal of Obstetrics & Gynecology 1982;143(6):693-9.
51. Hack M, Merkatz I, McGrath S, Jones P, Fanaroff A. Catch-up growth in very-low-birth-weight infants. Clinical correlates. American Journal of Diseases of Children 1984;138(4):370-5.

52. Lucas A, Morley R, Cole T, Gore S. A randomised multicentre study of human milk versus formula and later development in preterm infants. Archives of Disease in Childhood Fetal & Neonatal Edition 1994;70(2):F141-6.
53. Morley R, Lucas A. Randomized diet in the neonatal period and growth performance until 7.5-8 y of age in preterm children. American Journal of Clinical Nutrition 2000;71(3):822-8.
54. Anderson J, Johnstone B, Remley D. Breast-feeding and cognitive development: a meta-analysis. American Journal of Clinical Nutrition 1999;70(4):525-35.
55. Committee on Nutrition of the Preterm Infant ESPGN. Nutrition and feeding of preterm infants. Acta Paediatrica Scandinavica - Supplement 1987;336:1-14.
56. Canadian Paediatric Society Committee on Nutrition. Nutrient needs and feeding of premature infants. Canadian Medical Association Journal 1995;152(11):1765-85.
57. Tsang R, Uauy R, Koletzko B, Zlotkin S, eds. Nutrition of the Preterm Infant. Scientific Basis and Practical Guidelines. 2nd ed. Cincinnati: Digital Education Publishing Inc, 2005.
58. Kuschel C, Harding J. Protein supplementation of human milk for promoting growth in preterm infants. Cochrane Database of Systematic Reviews 2000;2.
59. Kuschel C, Harding J. Fat supplementation of human milk for promoting growth in preterm infants. Cochrane Database of Systematic Reviews 2000;2.
60. Kuschel C, Harding J. Carbohydrate supplementation of human milk to promote growth in preterm infants. Cochrane Database of Systematic Reviews 2000;2.
61. Kuschel C, Harding J. Multicomponent fortified human milk for promoting growth in preterm infants.[update of Cochrane Database Syst Rev. 2000;(2):CD000343; PMID: 10796349]. Cochrane Database of Systematic Reviews 2004;1.
62. Ronnholm KA, Sipila I, Siimes MA. Human milk protein supplementation for the prevention of hypoproteinemia without metabolic imbalance in breast milk-fed, very low-birth-weight infants. Journal of Pediatrics 1982;101(2):243-7.
63. Putet G, Rigo J, Salle B, Senterre J. Supplementation of pooled human milk with casein hydrolysate: energy and nitrogen balance and weight gain composition in very low birth weight infants. Pediatric Research 1987;21(5):458-61.
64. Polberger SK, Axelsson IA, Raiha NC. Growth of very low birth weight infants on varying amounts of human milk protein. Pediatric Research 1989;25(4):414-9.
65. Boehm G, Muller D, Beyreiss K, Raiha N. Evidence for functional immaturity of the ornithine-urea cycle in very-low-birth-weight infants. 1988.
66. Modanlou H, Lim M, Hansen J, Sickles V. Growth, biochemical status, and mineral metabolism in very-low-birth-weight infants receiving fortified preterm human milk. Journal of Pediatric Gastroenterology & Nutrition 1986;5(5):762-7.

67. Carey D, Rowe J, Goetz C, Horak E, Clark R, Goldberg B. Growth and phosphorus metabolism in premature infants fed human milk, fortified human milk, or special premature formula. Use of serum procollagen as a marker of growth. American Journal of Diseases of Children 1987;141(5):511-5.
68. Greer F, McCormick A. Improved bone mineralization and growth in premature infants fed fortified own mother's milk. Journal of Pediatrics 1988;112(6):961-9.
69. Pettifor J, Rajah R, Venter A, Moodley G, Opperman L, Cavaleros M, Ross F. Bone mineralization and mineral homeostasis in very low-birth-weight infants fed either human milk or fortified human milk. Journal of Pediatric Gastroenterology & Nutrition 1989;8(2):217-24.
70. Kashyap S, Schulze K, Forsyth M, Dell R, Ramakrishnan R, Heird W. Growth, nutrient retention, and metabolic response of low-birth-weight infants fed supplemented and unsupplemented preterm human milk. American Journal of Clinical Nutrition 1990;52(2):254-62.
71. Zuckerman M, Pettifor JM. Rickets in very-low-birth-weight infants born at Baragwanath Hospital. South African Medical Journal Suid-Afrikaanse Tydskrif Vir Geneeskunde 1994;84(4):216-20.
72. Lucas A, Fewtrell M, Morley R, Lucas P, Baker B, Lister G, Bishop N. Randomized outcome trial of human milk fortification and developmental outcome in preterm infants. American Journal of Clinical Nutrition 1996;64(2):142-51.
73. Wauben I, Atkinson S, Grad T, Shah J, Paes B. Moderate nutrient supplementation of mother's milk for preterm infants supports adequate bone mass and short-term growth: a randomized, controlled trial. American Journal of Clinical Nutrition 1998;67(3):465-72.
74. Nicholl R, Gamsu H. Changes in growth and metabolism in very low birthweight infants fed with fortified breast milk. Acta Paediatrica 1999;88(10):1056-61.
75. Faerk J, Petersen S, Peitersen B, Michaelsen K. Diet and bone mineral content at term in premature infants. Pediatric Research 2000;47(1):148-56.
76. Gross S. Bone mineralization in preterm infants fed human milk with and without mineral supplementation. Journal of Pediatrics 1987;111(3):450-8.
77. Tudehope DI, Mitchell F, Cowley DM. A comparative study of a premature infant formula and preterm breast milk for low birthweight infants. Australian Paediatric Journal 1986;22(3):199-205.
78. Roberts SB, Lucas A. Energetic efficiency and nutrient accretion in preterm infants fed extremes of dietary intake. Human Nutrition - Clinical Nutrition 1987;41(2):105-13.
79. Putet G, Senterre J, Rigo J, Salle B. Nutrient balance, energy utilization, and composition of weight gain in very-low-birth-weight infants fed pooled human milk or a preterm formula. Journal of Pediatrics 1984;105(1):79-85.
80. Brooke O, Wood C, Barley J. Energy balance, nitrogen balance, and growth in preterm infants fed expressed breast milk, a premature infant formula, and two low-solute adapted formulae. Archives of Disease in Childhood 1982;57(12):898-904.

81. Svenningsen NW, Lindroth M, Lindquist B. A comparative study of varying protein intake in low birthweight infant feeding. Acta Paediatrica Scandinavica Supplement 1982;296:28-31.
82. Davies D. Adequacy of expressed breast milk for early growth of preterm infants. Archives of Disease in Childhood 1977;52(4):296-301.
83. Schanler R, Shulman R, Lau C. Feeding strategies for premature infants: beneficial outcomes of feeding fortified human milk versus preterm formula. Pediatrics 1999;103(6 Pt 1):1150-7.
84. Raschko PK, Hiller JL, Benda GI, Buist NR, Wilcox K, Reynolds JW. Nutritional balance studies of VLBW infants fed their mothers' milk fortified with a liquid human milk fortifier. Journal of Pediatric Gastroenterology & Nutrition 1989;9(2):212-8.
85. Kashyap S, Schulze K, Forsyth M, Zucker C, Dell R, Ramakrishnan R, Heird W. Growth, nutrient retention, and metabolic response in low birth weight infants fed varying intakes of protein and energy. Journal of Pediatrics 1988;113(4):713-21.
86. Wauben I, Westerterp K, Gerver WJ, Blanco C. Effect of varying protein intake on energy balance, protein balance and estimated weight gain composition in premature infants. European Journal of Clinical Nutrition 1995;49(1):11-6.
87. Schulze KF, Stefanski M, Masterson J, Spinnazola R, Ramakrishnan R, Dell RB, Heird WC. Energy expenditure, energy balance, and composition of weight gain in low birth weight infants fed diets of different protein and energy content. Journal of Pediatrics 1987;110(5):753-9.
88. Goldman H, Freudenthal R, Holland B, Karelitz S. Clinical effects of two different levels of protein intake on low-birth-weight infants. Journal of Pediatrics 1969;74(6):881-9.
89. Kashyap S, Forsyth M, Zucker C, Ramakrishnan R, Dell R, Heird W. Effects of varying protein and energy intakes on growth and metabolic response in low birth weight infants. Journal of Pediatrics 1986;108(6):955-63.
90. Embleton N, Cooke R. Protein requirements in preterm infants: Effect of different levels of protein intake on growth and body composition. Pediatric Research 2005;58(5):855 - 60.
91. Carlson S, Ziegler E. Nutrient intakes and growth of very low birth weight infants. Journal of Perinatology 1998;18(4):252-8.
92. Radmacher P, Looney S, Rafail S, Adamkin D. Prediction of extrauterine growth retardation (EUGR) in VVLBW infants. Journal of Perinatology 2003;23(5):392-5.
93. Premji S, Paes B, Jacobson K, Chessell L. Evidence-based feeding guidelines for very low-birth-weight infants. Advances in Neonatal Care 2002;2(1):5-18.
94. Smith J. Early enteral feeding for the very low birth weight infant: the development and impact of a research-based guideline. Neonatal Network - Journal of Neonatal Nursing 2005;24(4):9-19.
95. Dept Neonatal Medicine Protocol Book Royal Prince Alfred Hospital. Internet: http://www.cs.nsw.gov.au/rpa/neonatal/ (accessed 22/12/2005).
96. Carlson S, Ziegler E. Feeding. Edtion ed. In: Bell E, Segar J, eds. Iowa Neonatology Handbook: Electronic resource, 1994.

97. Tyson JE, Kennedy KA. Trophic feedings for parenterally fed infants.[update of Cochrane Database Syst Rev. 2000;(2):CD000504; PMID: 10796374]. Cochrane Database of Systematic Reviews 2005(3):CD000504.
98. American Academy of Pediatrics. Breastfeeding and the use of human milk Pediatrics 1997;100(6):1035-9.
99. The Royal Autstralasian College of Physicians Paediatrics & Child Health Division. Paediatric Policy: Breastfeeding. RACP website, 2002.
100. Lucas A, Morley R, Cole T, Lister G, Leeson-Payne C. Breast milk and subsequent intelligence quotient in children born preterm. Lancet 1992;339(8788):261-4.
101. Brooke O, Onubogu O, Heath R, Carter N. Human milk and preterm formula compared for effects on growth and metabolism. Archives of Disease in Childhood 1987;62(9):917-23.
102. Cooper P, Rothberg A, Pettifor J, Bolton K, Devenhuis S. Growth and biochemical response of premature infants fed pooled preterm milk or special formula. Journal of Pediatric Gastroenterology & Nutrition 1984;3(5):749-54.
103. Atkinson S, Bryan M, Anderson G. Human milk feeding in premature infants: protein, fat, and carbohydrate balances in the first two weeks of life. Journal of Pediatrics 1981;99(4):617-24.
104. Stein H, Cohen D, Herman AA, Rissik J, Ellis U, Bolton K, Pettifor J, MacDougall L. Pooled pasteurized breast milk and untreated own mother's milk in the feeding of very low birth weight babies: a randomized controlled trial. Journal of Pediatric Gastroenterology & Nutrition 1986;5(2):242-7.
105. Atkinson S, Radde I, Anderson G. Macromineral balances in premature infants fed their own mothers' milk or formula. Journal of Pediatrics 1983;102(1):99-106.
106. Lucas A, Brooke O, Baker B, Bishop N, Morley R. High alkaline phosphatase activity and growth in preterm neonates. Archives of Disease in Childhood 1989;64(7 Spec No):902-9.
107. Schanler R. The use of human milk for premature infants. Pediatric Clinics of North America 2001;48(1):207-19.
108. Ronnholm KA, Perheentupa J, Siimes MA. Supplementation with human milk protein improves growth of small premature infants fed human milk. Pediatrics 1986;77(5):649-53.
109. Kuschel C, Harding J. Multicomponent fortified human milk for promoting growth in preterm infants.[update in Cochrane Database Syst Rev. 2004;(1):CD000343; PMID: 14973953]. Cochrane Database of Systematic Reviews 2000;2.
110. Ziegler E. Breast-milk fortification. Acta Paediatrica 2001;90(7):720-3.
111. Atkinson S, Bryan M, Anderson G. Human milk: difference in nitrogen concentration in milk from mothers of term and premature infants. Journal of Pediatrics 1978;93(1):67-9.
112. Gross S, David R, Bauman L, Tomarelli R. Nutritional composition of milk produced by mothers delivering preterm. Journal of Pediatrics 1980;96(4):641-4.

113. Sann L, Bienvenu F, Lahet C, Bienvenu J, Bethenod M. Comparison of the composition of breast milk from mothers of term and preterm infants. Acta Paediatrica Scandinavica 1981;70(1):115-6.
114. Gross S, Geller J, Tomarelli R. Composition of breast milk from mothers of preterm infants. Pediatrics 1981;68(4):490-3.
115. Anderson G, Atkinson S, Bryan M. Energy and macronutrient content of human milk during early lactation from mothers giving birth prematurely and at term. American Journal of Clinical Nutrition 1981;34(2):258-65.
116. Lemons J, Moye L, Hall D, Simmons M. Differences in the composition of preterm and term human milk during early lactation. Pediatric Research 1982;16(2):113-7.
117. Chandra R. Immunoglobulin and protein levels in breast milk produced by mothers of preterm infants. Nutrition Research 1982;2:27 - 30.
118. Anderson D, Williams F, Merkatz R, Schulman P, Kerr D, Pittard W, 3rd. Length of gestation and nutritional composition of human milk. American Journal of Clinical Nutrition 1983;37(5):810-4.
119. Butte N, Garza C, Johnson C, Smith E, Nichols B. Longitudinal changes in milk composition of mothers delivering preterm and term infants. Early Human Development 1984;9(2):153-62.
120. Saarela T, Kokkonen J, Koivisto M. Macronutrient and energy contents of human milk fractions during the first six months of lactation. Acta Paediatrica 2005;94(9):1176-81.
121. Schanler RJ, Oh W. Composition of breast milk obtained from mothers of premature infants as compared to breast milk obtained from donors. Journal of Pediatrics 1980;96(4):679-81.
122. Lepage G, Collet S, Bougle D, Kien L, Lepage D, Dallaire L, Darling P, Roy C. The composition of preterm milk in relation to the degree of prematurity. American Journal of Clinical Nutrition 1984;40(5):1042-9.
123. Lucas A, Hudson G. Preterm milk as a source of protein for low birthweight infants. Archives of Disease in Childhood 1984;59(9):831-6.
124. Atkinson S, Lonnerdal B. Nonprotein nitrogen fractions of human milk. Edtion ed. In: Jenson R, ed. Handbook of Milk Composition. San Diego: Academic Press, INC., 1995:369 - 87.
125. Atkinson S, Anderson G, Bryan M. Human milk: comparison of the nitrogen composition in milk from mothers of premature and full-term infants. American Journal of Clinical Nutrition 1980;33(4):811-5.
126. Polberger S, Raiha NC, Juvonen P, Moro GE, Minoli I, Warm A. Individualized protein fortification of human milk for preterm infants: comparison of ultrafiltrated human milk protein and a bovine whey fortifier. Journal of Pediatric Gastroenterology & Nutrition 1999;29(3):332-8.
127. Arslanoglu S, Moro G, Ziegler E. Adjustable fortification of human milk fed to preterm infants: does it make a difference? Journal of Perinatalogy 2006;26:614 - 21.
128. Moro G, Minoli I, Ostrom M, Jacobs J, Picone T, Raiha N, Ziegler E. Fortification of human milk: evaluation of a novel fortification scheme and of a new fortifier. Journal of Pediatric Gastroenterology & Nutrition 1995;20(2):162-72.
129. Higgins J, Green S, (editors). Cochrane Handbook for Systematic Reviews of Interventions Version 5.0.0 ed: The Cochrane Collaboration, 2008.

130. Polberger S, Raiha N. Individualized fortification of human milk for very low birth weight infants: milk protein and energy analyses for improved nutritional management. Pediatric Research 1995;37:316A.
131. Gathwala G, Chawla M, Gehlaut V. Fortified human milk in the small for gestational age neonate. Indian Journal of Pediatrics 2007;74(9):815-8.
132. Mukhopadhyay K, Narnag A, Mahajan R. Effect of human milk fortification in appropriate for gestation and small for gestation preterm babies: a randomized controlled trial. Indian Pediatrics 2007;44(4):286-90.
133. Polberger SK, Axelsson IE, Raiha NC. Amino acid concentrations in plasma and urine in very low birth weight infants fed protein-unenriched or human milk protein-enriched human milk. Pediatrics 1990;86(6):909-15.
134. Gathwala G, Shaw C, Shaw P, Yadav S, Sen J. Human milk fortification and gastric emptying in the preterm neonate. International Journal of Clinical Practice 2008;62(7):1039-43.
135. O'Connor DL, Khan S, Weishuhn K, Vaughan J, Jefferies A, Campbell DM, Asztalos E, Feldman M, Rovet J, Westall C, Whyte H. Growth and nutrient intakes of human milk-fed preterm infants provided with extra energy and nutrients after hospital discharge. Pediatrics 2008;121(4):766-76.
136. Schanler RJ, Abrams SA. Postnatal attainment of intrauterine macromineral accretion rates in low birth weight infants fed fortified human milk. Journal of Pediatrics 1995;126(3):441-7.
137. Berseth C, Van Aerde J, Gross S, Stolz S, Harris C, Hansen J. Growth, efficacy, and safety of feeding an iron-fortified human milk fortifier. Pediatrics 2004;114(6).
138. Wauben I, Gibson R, Atkinson S. Premature infants fed mothers' milk to 6 months corrected age demonstrate adequate growth and zinc status in the first year. Early Human Development 1999;54(2):181-94.
139. Noack J, Boehm G. Influence of casein feeding to very-low-birth weight infants on the plasma amino acid concentrations and the renal amino acid excretion. Biomedica Biochimica Acta 1991;50(12):1241-9.
140. Doege C, Bauer J. Effect of high volume intake of mother's milk with an individualized supplementation of minerals and protein on early growth of preterm infants <28 weeks of gestation. Clinical Nutrition 2007;26(5):581-8.
141. Ronnholm KA, Simell O, Siimes MA. Human milk protein and medium-chain triglyceride oil supplementation of human milk: plasma amino acids in very low-birth-weight infants. Pediatrics 1984;74(5):792-9.
142. Boehm G, Senger H, Friedrich M, Muller D, Beyreiss K. Protein supplementation of human milk for the nutrition of VLBW-infants: human milk protein vs. meat protein hydrolysate. Klinische Padiatrie 1990;202(5):316-20.
143. Boehm G, Borte M, Muller D, Senger H, Rademacher C. [Neonatal nutrition with enriched human milk. EOPROTIN 60 in comparison with human albumin]. Kinderarztliche Praxis 1991;59(10):293-8.
144. Boehm G, Borte M, Bellstedt K, Moro G, Minoli I. Protein quality of human milk fortifier in low birth weight infants: effects on growth and plasma amino acid profiles. European Journal of Pediatrics 1993;152(12):1036-9.

145. Hagelberg S, Lindblad B, Persson B. Amino acid levels in the critically ill preterm infant given mother's milk fortified with protein from human or cow's milk. Acta Paediatrica Scandinavica 1990;79(12):1163-74.
146. Moro G, Minoli I, Fulconis F, Clementi M, Raiha N. Growth and metabolic responses in low-birth-weight infants fed human milk fortified with human milk protein or with a bovine milk protein preparation. Journal of Pediatric Gastroenterology & Nutrition 1991;13(2):150-4.
147. Arslanoglu S, Moro G, Ziegler E. Preterm infants fed fortified human milk receive less protein than they need. Journal of Perinatology 2009;29(7):489-92.
148. Metcalf R, Dilena B, Gibson R, Marshall P, Simmer K. How appropriate are commercially available human milk fortifiers? Journal of Paediatrics & Child Health 1994;30(4):350-5.
149. Sankaran K, Papageorgiou A, Ninan A, Sankaran R. A randomized, controlled evaluation of two commercially available human breast milk fortifiers in healthy preterm neonates. Journal of the American Dietetic Association 1996;96(11):1145-9.
150. Reis BB, Hall RT, Schanler RJ, Berseth CL, Chan G, Ernst JA, Lemons J, Adamkin D, Baggs G, O'Connor D. Enhanced growth of preterm infants fed a new powdered human milk fortifier: A randomized, controlled trial. Pediatrics 2000;106(3):581-8.
151. Porcelli P, Schanler R, Greer F, Chan G, Gross S, Mehta N, Spear M, Kerner J, Euler AR. Growth in human milk-Fed very low birth weight infants receiving a new human milk fortifier. Annals of Nutrition & Metabolism 2000;44(1):2-10.
152. Zuppa AA, Girlando P, Scapillati ME, Maggio L, Romagnoli C, Tortorolo G. [Effects on growth, tolerability and biochemical parameters of two different human milk fortifiers in very low birth-weight newborns]. Pediatria Medica e Chirurgica 2004;26(1):45-9.
153. Eggum B. Blood urea measurement as a technique for assessing protein quality. British Journal of Nutrition 1970;24(4):983-8.
154. Polberger SK, Axelsson IE, Raiha NC. Urinary and serum urea as indicators of protein metabolism in very low birthweight infants fed varying human milk protein intakes. Acta Paediatrica Scandinavica 1990;79(8-9):737-42.
155. Davies D, Saunders R. Blood urea. Normal values in early infancy related to feeding practices. Archives of Disease in Childhood 1973;48(7):563-5.
156. Avery M, Clow C, Menkes J, Ramos A, Scriver C, Stern L, Wasserman B. Transient tyrosinemia of the newborn: dietary and clinical aspects. Pediatrics 1967;39(3):378-84.
157. International Dairy Federation. Standard 141C:2000 - Whole milk - Determination of milk fat, protein and lactose content - Guide for the operation of mid-infra-red instruments. Brussels: International Dairy Federation, 2000.
158. Michaelsen K, Pedersen S, Skafte L, Jaeger P, Peitersen B. Infrared analysis for determining macronutrients in human milk. Journal of Pediatric Gastroenterology & Nutrition 1988;7(2):229-35.
159. Faerk J, Skafte L, Petersen S, Peitersen B, Michaelsen K. Macronutrients in milk from mothers delivering preterm. Advances in Experimental Medicine & Biology 2001;501:409-13.

160. ICH Expert Working Group. ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology Q2 (R1). 1995.
161. Foss. Internet:http://www.foss.dk/Solutions/ProductsDirect/MilkoScanMinor/Technology.aspx (accessed 10/12/2006).
162. Fomon S. Requirements and recommended dietary intakes of protein during infancy. Pediatric Research 1991;30(5):391-5.
163. Standards Australia. AS 2300.1.2.1 Methods of chemical and pysical testing for the dairying industry. Method 1.2.1: General methods and principles - Determination of nitrogen - Reference Kjeldahl method. Sydney: Standards Australia, 1991.
164. Standards Australia. AS 2300.1.2.2-1988 Methods of chemical and physical testing for the dairying industry - General methods and principles - Determination of nitrogen - Nitrogen fractions from milk. Sydney: Standards Australia, 1988.
165. Newburg D, Neubauer S. Carboydrates in Milks: Analysis, Quantities, and Significance. Edtion ed. In: Jensen R, ed. Handbook of Milk Composition. Connecticut: Academic Press, 1995:273 - 350.
166. McVeagh P, Miller J. Human milk oligosaccharides: only the breast. Journal of Paediatrics & Child Health 1997;33(4):281-6.
167. Standards Australia. AS2300.1.3 Methods of chemical and physical testing for the dairying Industry - General methods and principles - Determination of fat - Gravimetric Method. Sydney: Standards Australia, 1988.
168. Bauer K, Bovermann G, Roithmaier A, Gotz M, Proiss A, Versmold HT. Body composition, nutrition, and fluid balance during the first two weeks of life in preterm neonates weighing less than 1500 grams. Journal of Pediatrics 1991;118(4 Pt 1):615-20.
169. World Health Organisation. WHO Child Growth Standards, Training course on child growth assessment. 2006. Internet: http://www.who.int/childgrowth/training/en/ accessed 12/07/07).
170. de Onis M, Onyango AW, Van den Broeck J, Chumlea WC, Martorell R. Measurement and standardization protocols for anthropometry used in the construction of a new international growth reference. Food & Nutrition Bulletin 2004;25(1):S27-36.
171. Donoghue D. Australian and New Zealand neonatal network 1998. In: Australian Institute of Health and Welfare NPSU, ed. Syndey, 2000.
172. Altman D, Schulz K, Moher D, Egger M, Davidoff F, Elbourne D, Gotzsche P, Lang T, CONSORT GROUP CSoRT, Review. The revised CONSORT statement for reporting randomized trials: explanation and elaboration.[see comment]. Annals of Internal Medicine 2001;134(8):663-94.
173. Moher D, Schulz K, Altman D. The CONSORT statement: revised recommendations for improving the quality of reports of parallel-group randomised trials. Lancet 2001;357(9263):1191-4.
174. Zwarenstein M, Treweek S, Gagnier JJ, Altman DG, Tunis S, Haynes B, Oxman AD, Moher D, Zwarenstein M, Treweek S, Gagnier JJ, Altman DG, Tunis S, Haynes B, Oxman AD, Moher D. Improving the reporting of pragmatic trials: an extension of the CONSORT statement. BMJ 2008;337:a2390.

175. McLennan W. Australian Standard Classification of Occupations. In: Statisitics AB, ed. Second Edition ed. Canberra: Australian Government Publishing Services, 1997.
176. Beeby P, Bhutap T, Taylor L. New South Wales population-based birthweight percentile charts. Journal of Paediatrics & Child Health 1996;32(6):512-8.
177. Roberts CL, Lancaster PA. National birthweight percentiles by gestational age for twins born in Australia. Journal of Paediatrics & Child Health 1999;35(3):278-82.
178. Mueller W, Martorell R. Reliability and accuracy of measurment. Edtion ed. In: Lohman T, Roche A, Martorell R, eds. Anthropometric Standardization Reference Manual. Champaign: Human Kinetics Books, 1988:83 - 6.
179. WHO Multicentre Growth Reference Study Group. Reliability of anthropometric measurements in the WHO Multicentre Growth Reference Study. Acta Paediatrica Supplement 2006;450:38-46.
180. Kramer M, Platt R, Wen S, Joseph K, Allen A, Abrahamowicz M, Blondel B, Breart G, Fetal/Infant Health Study Group of the Canadian Perinatal Surveillance System. A new and improved population-based Canadian reference for birth weight for gestational age. Pediatrics 2001;108(2):E35.
181. Flay BR. Efficacy and effectiveness trials (and other phases of research) in the development of health promotion programs. Preventive Medicine 1986;15(5):451-74.
182. Kronmal R. Spurious Correlation and the Fallacy of the Ratio Standard Revisited. Journal of the Royal Statistical Society 1993;156(3).
183. Berkman D, Lescano A, Gilman R, Lopez S, Black M, Berkman DS, Lescano AG, Gilman RH, Lopez SL, Black MM. Effects of stunting, diarrhoeal disease, and parasitic infection during infancy on cognition in late childhood: a follow-up study. Lancet 2002;359(9306):564-71.
184. Grantham-McGregor S, Cheung Y, Cueto S, Glewwe P, Richter L, Strupp B, International Child Development Steering G, Grantham-McGregor S, Cheung YB, Cueto S, Glewwe P, Richter L, Strupp B. Developmental potential in the first 5 years for children in developing countries. Lancet 2007;369(9555):60-70.
185. Mendez M, Adair L. Severity and timing of stunting in the first two years of life affect performance on cognitive tests in late childhood. Journal of Nutrition 1999;129(8):1555-62.
186. Walker SP, Chang SM, Powell CA, Grantham-McGregor SM, Walker SP, Chang SM, Powell CA, Grantham-McGregor SM. Effects of early childhood psychosocial stimulation and nutritional supplementation on cognition and education in growth-stunted Jamaican children: prospective cohort study. Lancet 2005;366(9499):1804-7.
187. Bhutta A, Cleves M, Casey P, Cradock M, Anand K, Bhutta AT, Cleves MA, Casey PH, Cradock MM, Anand K. Cognitive and behavioral outcomes of school-aged children who were born preterm: a meta-analysis. JAMA 2002;288(6):728-37.
188. Stephens BE, Vohr BR, Stephens BE, Vohr BR. Neurodevelopmental outcome of the premature infant. Pediatric Clinics of North America 2005;56(3):631-46.

189. Taylor HG, Klein N, Hack M. School-age consequences of birth weight less than 750 g: a review and update. Developmental Neuropsychology 2000;17(3):289-321.
190. De Curtis M, Candusso M, Pieltain C, Rigo J. Effect of fortification on the osmolality of human milk. Archives of Disease in Childhood Fetal & Neonatal Edition 1999;81(2):F141-3.
191. Cooke R, Griffin I, McCormick K, Wells J, Smith J, Robinson S, Leighton M. Feeding preterm infants after hospital discharge: effect of dietary manipulation on nutrient intake and growth. Pediatric Research 1998;43(3):355-60.
192. Cooke R. Adjustable fortification of human milk fed to preterm infants. Journal of Perinatology 2006;26(10):591-2.
193. de Boo H, Harding J. Protein metabolism in preterm infants with particular reference to intrauterine growth restriction. Archives of Disease in Childhood Fetal & Neonatal Edition 2007;92(4).
194. Thom H, McKay E, Gray DW. Protein concentrations in the umbilical cord plasma of premature and mature infants. Clinical Science 1967;33(3):433-44.
195. Cartlidge P, Rutter N. Serum albumin concentrations and oedema in the newborn. Archives of Disease in Childhood 1986;61(7):657-60.
196. Australian and New Zealand Neonatal Network. Report of the Australian and New Zealand Neonatal Network 2006. Sydney, 2009.
197. Quan R, Yang C, Rubinstein S, Lewiston NJ, Stevenson DK, Kerner JA, Jr. The effect of nutritional additives on anti-infective factors in human milk. Clinical Pediatrics 1994;33(6):325-8.