Title: Comparing different methods of human breast milk fortification using measured versus assumed macronutrient composition to target reference growth: a RCT

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Abbreviations:

AGA: appropriate for gestational age
PMA: postmenstrual age
DHM: donor human milk
ELBW: extremely low birth weight
EN: enteral nutrition
FFM: fat free mass
FM: fat mass
GpA: assumed milk composition group
GpM: measured milk composition group
HM: human milk
HMA: human milk analyser
HMF: human milk fortifier
KEMH: King Edward Memorial Hospital
MEF: minimal enteral feeds
MOM: mothers own milk

NEC: necrotising enterocolitis

PER: protein energy ratio

PN: parenteral nutrition

Running Title: Comparing two methods of milk fortification

Keywords: protein intake; protein energy ratio; body composition; human milk fortification

ABSTRACT

The variable content of human breast milk suggests its routine fortification may result in sub-optimal nutritional intakes and growth. In a pragmatic trial, we randomised infants born <30 weeks gestation to either the intervention (Igp) of fortifying milk on measured composition according to birth weight criteria and postmenstrual age or our routine practice (RPgp), of fortifying on assumed milk composition to target 3.8-4.4 g protein/kg/d and 545-629 kJ/kg/d. Milk composition was measured with MIRIS™. Percentage fat mass (%FM) was measured with PEAPOD™. Effect of macronutrient intakes and clinical variables on growth was assessed using mixed model analysis. Mean measured protein content (1.6 g/100 mL) was higher than the assumed value (1.4 g 100/mL), often leading to lower amounts of fortifier added to the milk of intervention infants. At discharge [Igp *vs.* RPgp], total protein [3.2 (0.3) g *vs*. 3.4 (0.4) g, p=0.067] and energy [456 (39) kJ *vs.* 481 (48) kJ, p=0.079] intakes from all nutrition sources, weight gain velocity [11.4 (1.4) *vs.* 12.1 (1.6) g/kg/d, p=0.135] and %FM [13.7 (3.6) *vs.*13.6 (3.5) %, p=0.984] did not significantly differ between groups. A protein intake >3.4 g/kg/d reduced %FM by 2%. Nutrition and growth was not improved by targeting milk fortification according to birth weight criteria and postmenstrual age using measured milk composition, compared to routine practice. Targeting fortification on measured composition is labor intensive, requiring frequent milk sampling and precision measuring equipment, perhaps reasons for its limited practice. Guidance around safe upper levels of milk fortification is needed. The trial was registered with the Australian New Zealand Clinical Registry (http://www.anzctr.org.au) as ACTRN12610000443099.

INTRODUCTION

In the past decade and a half, there has been universal recognition that increased amounts of protein and energy are necessary to address the nutrition and growth deficits that accrue postnatally in preterm infants. In attempts to address this, parenteral amino acids and lipid are delivered earlier and more aggressively after preterm birth, and as well, the protein and energy content of breast milk is increased through the routine addition of human breast milk fortifier, using an assumed milk composition. Despite these strategies, preterm infants often receive less protein and energy than they need[1], which is concerning, since growth and composition of weight gain can be influenced by the macronutrient composition of the diet[2,3] and recent studies suggest that at term equivalent age, preterm infants have altered and increased adiposity and increased amounts of ectopic lipid, compared to their peers[4,5].

Whilst international consensus guidelines suggest energy intakes should be based on birth weight

criteria (<1000 g: 545-629 kJ/kg/d; 1000 g to <1500 g: 461-545 kJ) and recommend reducing protein with increasing postmenstrual age (PMA)[6], European guidelines base protein recommendation on body weight and suggest energy intakes exceeding 565 kJ/kg/d (135 kcal/kg/d) may promote fat deposition rather than accretion of lean tissue[7] (Table 1).

Attempts to improve nutritional and growth outcomes by trialling different fortification regimens have had mixed results[8-10]. Polberger et al.[8] fortified milk with human milk protein (n=16) or bovine fortifier (n=16) to provide a targeted protein intake of 3.5 g kg/kg/d, based on infant weight, volume intake (150 mL $kg^{-1}d^{-1}$ to 170 mL $kg^{-1}d^{-1}$), feed tolerance and measured protein content of the milk. They found no significant differences in growth or biochemical outcomes between groups. De Halleux and colleagues[9,11] measured milk composition and fortified the milk of 24 very low birth weight infants each for at least 3 weeks duration from 2006 through to 2011 using a bovine whey protein fortifier and a fat supplement and demonstrated less variation in protein intake than would be achieved theoretically with routine fortification. Arslanoglu et al.[10] did not measure milk composition but demonstrated that in adjusting fortifier on the basis of plasma urea using a commercially prepared, bovine-based fortifier, infants gained more weight than those who received routine fortification. However protein intakes for all infants were significantly and consistently lower than recommended intakes[1]. Some of these studies have been of short duration, a variety of products and methods have been used to fortify the milk, differences and improvements in growth outcomes have not been consistently shown and body composition, which has emerged as a necessary measure of nutrition adequacy, has not been assessed.

We conducted a randomised pragmatic study to test the hypothesis that growth and body composition of preterm infants more closely matches intrauterine growth[12,13] if fortification based on measured milk composition is used to target consensus protein intakes according to postmenstrual age and energy intakes according to birth weight (Table 1) rather than using an assumed milk composition to target upper limits of consensus protein (3.8-4.4 g/kg/d) and energy (545-629 kJ/kg/d) intakes[6].

MATERIALS AND METHODS

Recruitment Criteria

Forty infants born <30 weeks gestation admitted to the NICU at King Edward Memorial Hospital (KEMH) in Western Australia were recruited for the study period (January 2009 to June 2009) from birth to near term postmenstrual age if they were without congenital abnormalities, if maternal intention was to feed HM and if living remotely would not prevent participation at all assessments.

This age criteria was implemented because all infants born <30 w gestation admitted to the KEMH NICU are given parenteral nutrition as first fluids. At this gestation, a weight of 1000 g corresponds to the 10th percentile on Fenton's growth chart and our routine practice targets the consensus energy recommendation for infants weighing <1000 g at birth. The primary outcomes for this trial were weight, length, head circumference, weight gain velocity[14,15] and percentage fat mass measured using air displacement plethysmography (PEAPOD, LMI, Concord, CA, USA) at discharge/corrected term. The secondary outcomes reported elsewhere[16] were sub-cutaneous adipose and muscle tissue measurements taken by ultrasound. Transfer of infants (n=21) during the study from tertiary to Level 2 outlying nurseries within a 30 km radius of KEMH did not interrupt study protocol; infants continued in the trial to completion. Comparisons between 'transferred' *vs* 'not transferred' infants showed no baseline differences and no growth outcome differences within each treatment group. The Ethics Committees at both KEMH and The University of Western Australia reviewed and approved the study protocol. Written informed consent was obtained from the infants' mothers prior to commencing the study. The trial was registered with the Australian New Zealand Clinical Registry (http://www.anzctr.org.au) as ACTRN12610000443099; UTNU1111-1115-4183.

Randomisation and Blinding

Infants were randomised to the intervention (individualised fortification based on *measured* composition according to birth weight criteria and PMA), or routine practice (routinely optimised fortification based on *assumed* composition to target 3.8-44 g protein/kg/d; 545-629 kJ/kg/d (130-150 kcal/kg/d)), and the allocation ratio was 1:1. Twins were randomised as individuals. Randomisation sequence was achieved by random draw, without replacement (GM). Group allocation was concealed in sequentially numbered, opaque, sealed envelopes, which remained unopened until parental consent had been obtained and infant demographics had been recorded (research nurse or GM). Parents and the clinical teams managing the care of infants (including making the clinical decisions. prescribing each infant's daily fluid intake and fortification status and maintaining clinical records) were blinded to group allocations. Allocated nursing staff in the centralised milk room at KEMH, and in the outlying hospitals were responsible for collecting and freezing the milk samples prior to fortification and for adding fortifier to the milk as per study protocol. The chief investigator (GM) was responsible for (i) conducting the milk analysis; (ii) calculating the weekly mean composition of each infant's milk and the amount of fortifier to be added to the milk of intervention infants; (iii) providing fortification instructions to nursing staff for all study participants, (iv) data collection; and (v) ensuring study protocol was understood and maintained.

Milk Sampling and Analysis

A mother's milk was fed in the order in which it was expressed for at least the first 14 days of enteral feeding, and extending up to the first 28 days if available for infants born <26 weeks gestation. Any residual samples of early frozen milk not used prior to an infant receiving fresh milk were added to feeds over time, as needed. Therefore, each infant's daily native milk feed was made up from their own mothers' individual and pooled collections of expressed milk and may have included milk expressions from different days. A well-mixed sample (3-6 mL) of each infant's daily milk feed (MOM or DHM) was collected prior to fortification by nursing staff and labelled and frozen at minus 20^0 C (minus 5^0 C for outlying Level 2 nurseries). Each batch of weekly samples was gathered and analysed in the Human Milk Bank laboratory at the end of each week by GM. The samples were defrosted, warmed in a water bath to 40^0 C, homogenised (1.5 seconds per mL of sample; Sonics Vibracell, Model VCX-130, Sonics and Materials Inc, Newtown Ct, USA) and the protein, fat and lactose concentration determined using the MIRIS (human milk analyser: processes milk setting). The method has been described and evaluated elsewhere[17,18]. For each week that fortified feeds were prescribed for an infant, the weekly mean content of the infant's milk samples from the previous week was used to fortify the infant's milk feeds for the following week.

Fortification methods

A commercial multi-component HMF (Wyeth Nutritionals, Limerick, Ireland), a protein powder (Beneprotein, Novartis, Minneapolis, MN USA) and an energy supplement (Duocal, SHS International Limited, Liverpool, UK) were used to fortify milk feeds.

Milk for the intervention infants (Igp) was fortified with variable amounts of these fortifiers (HMF: maximum 4 g/100 mL; Beneprotein: maximum 0.5 g/100 mL; Duocal: maximum 3.0 g/100 mL), depending upon measured milk composition and fluid status (fluid restricted 130 to 150 mL/kg/d; non-fluid restricted 160 to 180 mL/kg/d). Protein was first adjusted, then if necessary, energy supplemented, to best achieve targets.

Milk for the infants fed according to routine practice (RPgp) was fortified in fixed dose amounts (HMF 4 g/100 mL for non-fluid restricted infants; HMF 4 g/100 mL + Beneprotein 0.5 g/100 mL + Duocal 2.5 g/100 mL for fluid restricted infants) using an assumed composition derived from published[19] and unpublished macronutrient milk analysis of preterm milk conducted in our Unit (protein 1.4%; fat 4.4%; lactose 6.8%). Our assumed composition falls within the published range of data describing preterm macronutrient composition of milk expressed over the first one to two months of lactation[20-23]

Nutrition

Until the initiation of fortified milk feeds, all infants were fed according to the Unit's standard feeding regimen:

Parenteral Nutrition (PN) On day one of life, 5% or 7.5% glucose and 1.5% amino acids were infused until day two, when individualised parenteral nutrition, including lipid (20%, 1.0 g $kg^{-1}d^{-1}$), electrolytes and micronutrients, was commenced. Subsequently, concentrations and rates were increased, targeting recommended intakes[6].

Enteral Nutrition (EN) Minimal enteral feeds (MEF), using frozen 'mothers' own milk' (MOM) in the sequence in which it was expressed, were initiated as early as possible after birth and increased, following a standardised regimen. Parenteral nutrition was simultaneously reduced. If MOM was unavailable, pasteurised donor human milk (DHM) was available for the infants until at least a post menstrual age of 34 weeks. As per unit guidelines and using clinical discretion, the medical teams prescribed and ceased fortification once enteral volumes ≥100 mL/kg/d were achieved.

Nutrition Intake during Hospital stay

All fluid and intake consumed during hospital stay were recorded retrospectively from the observation charts from midnight on day one of life until the discharge measurements. The milk volume consumed during a breastfeed was estimated to be equivalent to that amount normally given at a scheduled feed unless a top-up was also given, in which case the estimated volume taken at the breast was calculated by subtracting the volume of the top-up from the scheduled feed volume. Macronutrient intakes were calculated using composition data of daily milk feeds obtained for both groups with the HMA and energy intakes were derived using the Atwater values [kJ g^{-1} (kcal g^{-1})] for protein 16 (4), fat 37 (9) and lactose 16 (4) adopted by the National Health and Medical Research Council[24].

Body Composition

Percentage fat mass (%FM) measurements were taken at discharge (n=32) or on transfer to outlying Level 2 nurseries (n=8) using air displacement plethysmography (PEAPOD, LMI, Concord, CA, USA). The technical design and the methodology underpinning a PEAPOD measurement has been described elsewhere[25,26]. Briefly, the PEAPOD utilises the classic 2-compartment BC model to measure infants weighing between one and eight kilograms. Total body density is calculated from the direct measurements of body mass (electronic scale) and volume (air displacement). The software provided by LMI incorporates algorithms to derive percent body fat and FFM. These algorithms use the constant FM density value of 0.9007 g mL^{-1} and predetermined FFM density values modelled using the data of Fomon et al.[13].

Gestational Age and Post Menstrual Age

Gestational age was calculated as the time elapsed from date of the first day of the last menstrual period to delivery. If the date of the last menstrual period was unknown, the modified Ballard method was employed. Post menstrual age (PMA) was calculated according to the following formula: gestational age + postnatal age (time elapsed after birth).

Anthropometry

In accordance with the neonatal clinical care unit's (NCCU) measurement policy, weight, taken in the infant's incubator or with digital scales (g; SECA, Germany 10/20 kg; d = 5/10 g or PEAPOD), crown-heel length and occipital-frontal head circumference were measured at birth, discharge and at term PMA. Fenton's data were used to convert measurements to z-scores[27]. Infants requiring intensive care (NICU) were weighed daily and those in special care were weighed twice weekly, with daily weight derived by interpolation between each of the time-points. Weight gain velocity was calculated using an exponential model that has been validated in preterm infants[14,15].

Statistical Analysis

Descriptive statistics for continuous data were summarised using means and standard deviations or medians, interquartile ranges and ranges. Categorical data were summarised using frequency distributions. Univariate comparisons of continuous clinical data, nutritional intakes and anthropometric measures were conducted using one-sample t-tests, independent t-tests or Mann-Whitney tests according to normality, and Chi-square or Fisher exact tests were used for categorical comparisons. Reliability of %FM measurements for PEAPOD was determined by calculating the standard deviation, coefficient of variation and technical error. The technical error was defined as the $\sqrt{\Sigma d^2/2n}$, where *d* is the difference between two repeated tests for the paired observations. Comparisons of BC, measured as percentage body FM, were assessed at discharge using linear regression modelling after adjustment for postmenstrual age, weight z-score at measurement and residuals from a linear regression of length on weight at time of measurement. Linear mixed models analysis was conducted to produce growth curve models for weight gain velocity to discharge. A natural logarithm transformation was applied to the outcome to achieve normality, as indicated by residual diagnostics. Protein, carbohydrate and fat intakes and clinical variables were assessed for their effects on the rate of growth. Adjustment was made for birth weight z-score, postmenstrual age at time of measurement and chronological age. SAS 9.1 of the SAS System for Windows. Copyright © 2002-2010 SAS Institute Inc., Cary, NC, USA and PASW® 17 statistical software (SPSS Inc, Chicago, IL) were used for data analysis. All tests were two-tailed and p-values <0.05 were considered statistically significant.

Power

Estimation of sample size was based on data from a previous audit[28] using a mean growth rate of 12.8 g $kg^{-1}d^{-1}$ and standard deviation of 5 g $kg^{-1}d^{-1}$. A sample size of 20 in each group was sufficient to achieve 80% power to detect a difference of 3.4 g $kg^{-1}d^{-1}$ in a repeated measures design with an alpha level of 0.05 (Power Analysis and Sample Size (PASS) Statistical Software 2008).

RESULTS

Subject Demographics

Ninety-one infants admitted to the NCCU between 26 January and 9 June 2009 were born <30 weeks gestation. Fifty-one infants were excluded from the study for reasons including congenital renal abnormality (n=1), withheld consent (n=8), living remotely (n=13), paused recruitment (due to an investigator's absence) (n=19) and death (n=10) (Figure 2). Forty infants (Caucasian n=36, Australian Aboriginal: Igp n=1, RPgp n=1; Asian: RPgp n=1, Other: RP gp n=1) born from either singleton (n=24) or twin (n=16) births at a median (range) age of 27 (23-29) weeks and a birth weight of 1022 (480-1475) g were randomly assigned to the intervention (n=20) or routine practice (n=20). Siblings from two sets of twin births were each randomly allocated to the same group (n_1=both twins randomised to Igp; n_2=both twins randomised to RPgp), otherwise siblings were randomly allocated different groups. The clinical characteristics of the infants did not significantly differ between groups (Table 2). All infants were AGA (10^{th} percentile to 90^{th} percentile), with the exception of two infants fed routinely whose birth weights were on the 6^{th} weight percentile. The corrected PMA ranges of infants in the intervention and routine practice groups measured at discharge were 33-43 wk and 33-42 wk, respectively.

Composition of Milk Feeds

The mean (SD) measured protein, fat and lactose concentrations and the derived energy content and PER of milk feeds (n=1870 samples) were similar for both groups (Table 3).

Nutritional Intakes and Growth Outcomes

On average, 17% by volume of the total fluids received by infants while in hospital were given intravenously and human milk constituted 93% of the enteral intakes (84% MOM: Igp n=18, RPgp n=19; 16% DHM: Igp n=5, RPgp n=4), and an estimated 7% of MOM was breastfed. Unfortified milk feeds were suspended for one intervention infant for a period of 30 days when parenteral nutrition was provided due to suspected necrotising enterocolitis (Stage 2 NEC)[29]. Fortified feeds were prematurely ceased and not reinstituted for two intervention infants (feed intolerance n_1=day 15; n_2=day 47) and for two infants fed according to routine practice (n_1=Stage 2 NEC[29] day 14;

n_2=cow's milk protein intolerance day 32). One intervention infant did not receive fortifier and transitioned directly from unfortified milk to preterm formula. In total, 11 infants (Igp: n=6, RPgp: n=5) received some infant formula (IF) during their hospital stay. The number of days infants consumed fortified milk did not differ between groups (Igp: n=19: 44 (24) d *vs.* RPgp: n=20: 42 (23) d, p=0.801), and per study protocol, protein [3.2 (0.4) *vs.* 3.9 (0.3) g $kg^{-1}d^{-1}$, p<0.001] and energy [510 (39) *vs.* 559 (34) kJ $kg^{-1}d^{-1}$, p<0.001] intakes from fortified milk were lower in the intervention infants than in those fed according to routine practice (Table 3). Intention to treat analysis showed that post-fortification, (i.e. once infants made the initial transition from unfortified milk), total protein and energy intakes and the protein to energy ratio did not significantly differ between groups (Table 3). At discharge, weight (g), length (cm) head circumference (cm) and weight gain velocity were similar between groups (Table 4); eighteen infants had weights below the 10^{th} percentile (Igp: n=11, RPgp: n=7; p=0.204).

When adjusted for corrected postmenstrual age, chronological age and birth weight z-score, no significant difference in weight gain velocity was found between groups (p=0.140) (Table 5). There was an average 9% increase in weight gain velocity (g/kg/d) for every additional g/kg/d of enteral protein (95% CI 1% - 18%), p=0.024).

At discharge, rates of weight gain achieved by each group were significantly slower than the fetal rate (birth to discharge: Igp: p<0.001; RPgp: p<0.001; and after recovery of birth weight to discharge: Igp: p<0.001; RPgp p=0.051).

Body Composition

At discharge (Igp: 38 (2) weeks; RPgp: 38 (2) weeks), both groups had similar %FM, both univariately and after adjusting for PMA and length at measurement (p=0.269) (Table 5). Female infants had an average 3% greater FM than males (95% CI 1-5%), p<0.001). After inclusion of carbohydrate in the regression model, a protein intake > 3.4 g kg^{-1} d^{-1} (from all nutrition sources) reduced FM by 2% (p=0.042). The energy intakes of the 10 infants (Igp: n=4, RPgp: n=6; 25%) who consumed these higher protein intakes ranged between 389 and 537 kJ/kg/d, and their mean rate of weight gain, calculated after recovery of birth weight, was similar to the fetal rate (high protein: 14.7 g $kg^{-1}d^{-1}$ vs. fetal: 15 g $kg^{-1}d^{-1}$, p=0.514).

At discharge, preterm infants had a significantly greater mean %FM for age than the Reference Fetus (Igp: 13.7 (3.6)%; p<0.001, RPgp: 13.6 (3.5)%, p<0.001 *vs* Reference Fetus: 9.5%)[12].

DISCUSSION

The fortification design used in this pragmatic clinical trial did not improve growth or body

composition outcomes of infants born <30 weeks gestation compared to those fed according to routine practice. The method of fortifying human breast milk on measured composition, targeting consensus protein and energy intakes for corrected postmenstrual age and birth weight criteria was labour intensive and time consuming and did not prove a superior method over fortifying milk on assumed composition in fixed dose amounts to target upper consensus limits for the ELBW infant. There are mitigating reasons for these outcomes. Firstly, the milk protein content measured with MIRIS was higher (1.6 g) compared to the assumed value (1.4 g), and as a result, lower amounts of fortifier were often added to the milk of intervention infants. Two studies designed to evaluate the accuracy and precision of the MIRIS measurement have shown that the human milk analyser over-estimates milk protein by a small[17,18], but significant[18], amount, whilst a more recent study suggests it systematically underestimates protein content by a similar amount[30]. As the analytical methods and milk sampling designs differed in these studies, it is difficult to assess if there was need to make an adjustment to the measurement before calculating the amount of fortifier required to correct the protein deficit in the milk feeds of each of the infants in this trial. Menjo and colleagues[17] suggest this is a necessary strategy for clinicians, but it was not one adopted in this trial.

Another confounder in this study is that fortification was not adjusted to compensate for the dilutionary effect of breastfeeding (although contributing only an estimated 7% to milk intake), making continued titration of protein potentially superfluous after breastfeeding replaced one or more scheduled feeds. Furthermore, the maximum amounts of fortifier we arbitrarily permitted to be added to feeds sometimes proved limiting in achieving the desired level of fortification thought necessary to achieve the protein and energy and PER targets for an infant.

Since publication of the international consensus guidelines on which milk fortification in this study was designed[6], the Europeans have suggested that energy intakes should be maintained between 460 and 565 kJ $kg^{-1}d^{-1}$ (110 to 135 kcal $kg^{-1}d^{-1}$), and protein intakes and protein energy ratios should be based on a body weight range (<1000g: PER 3.6 to 4.1 and 1000 g – 1800 g: PER 3.2 to 3.6)[7]. These PER ranges, which are higher than the consensus targets[6] (2.8-3.3), are well above the PER (I: 2.7, RP: 2.9) that were achieved with milk fortification in this study. They are also higher than the PER achieved by Arslanoglu et al[10] who demonstrated that an extra 0.8 g of protein powder could be added to fortified milk feeds (HMF) without adverse clinical outcomes. However, using plasma urea to guide fortification still resulted in a range of protein and energy intakes (2.9-3.4 g $kg^{-1}d^{-1}$ and 535 kJ $kg^{-1}d^{-1}$)[1] below the consensus guidelines[6] and for protein at least, below the latest

ESPGHAN targets[7]. If the ESPGHAN recommendations[7] are to be achieved in practice, more studies are required to determine a safe, maximum level of fortification in the context of prescribed fluid intakes, variable milk composition, fortification practices, osmolality, feeding tolerance, risk of NEC and other clinical and metabolic outcomes.

Modelled fetal chemical data suggest the fetus more than doubles its %FM between 33 and 42 weeks (~7% to ~16%FM)[12,31]. After achieving mean protein intakes between 3.2 to 3.4 g $kg^{-1}d^{-1}$ and energy intakes between 460 and 480 kJ $kg^{-1}d^{-1}$ (PER 3.0), the mean %FM of the preterm infants in this study at the mean corrected postmenstrual age of 38 weeks was 13.7%. These BC data for preterm infants accord well with %FM estimates by Widdowson et al[31]. of the 38 week old fetus (12%) and estimates by Fomon et al. of the reference male (13.7%) and female (14.9%) term infants. Body composition and nutritional outcomes are difficult to measure in very preterm infants due to both the lack of suitable measuring methods in the neonatal setting and to the unpredictability of an infant's clinical condition. Measuring the true effect of different fortification regimens on growth outcomes is also logistically challenging because, despite randomisation, adjusting adequately for variations in metabolic and biological responses by individuals to feeding and clinical treatments is difficult. A larger sample size, routine measurement of breast milk transfer by test-weighing and non-intrusive and accurate bedside measurement methods for measuring growth and changes in BC from birth would assist in addressing these difficulties and were limitation of this study.

In this trial, the positive relationship between an achieved protein intake and %FM suggests that fortification regimens that target higher protein intakes may improve composition of growth, a concept that should form the basis of any future study design. Current human milk fortifiers are lacking sufficient protein to correct the deficit in breast milk that would facilitate the attainment of preterm nutrition and growth targets. Guidelines around safe upper limits of fortification are necessary to guide clinicians in their fortification practices. Fortifying breast milk using measured milk composition is time consuming and labour intensive, requiring precision equipment and the method may not be superior to routinely fortifying on assumed milk composition.

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Contributions to conception and design: GM, JS, KS, PEH
Acquisition of data: GM, DG
Analysis and interpretation of data: GM, JS, KS, PEH, EN, DG
Drafting the article GM
Revising it critically for intellectual content: JS, KS, PEH, EN, DG
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Conflict of Interest Statement

Sponsors and those who have been acknowledged had no role in study design, data collection, analysis or interpretation of data, writing of the manuscript or in the decision to submit the paper for publication.
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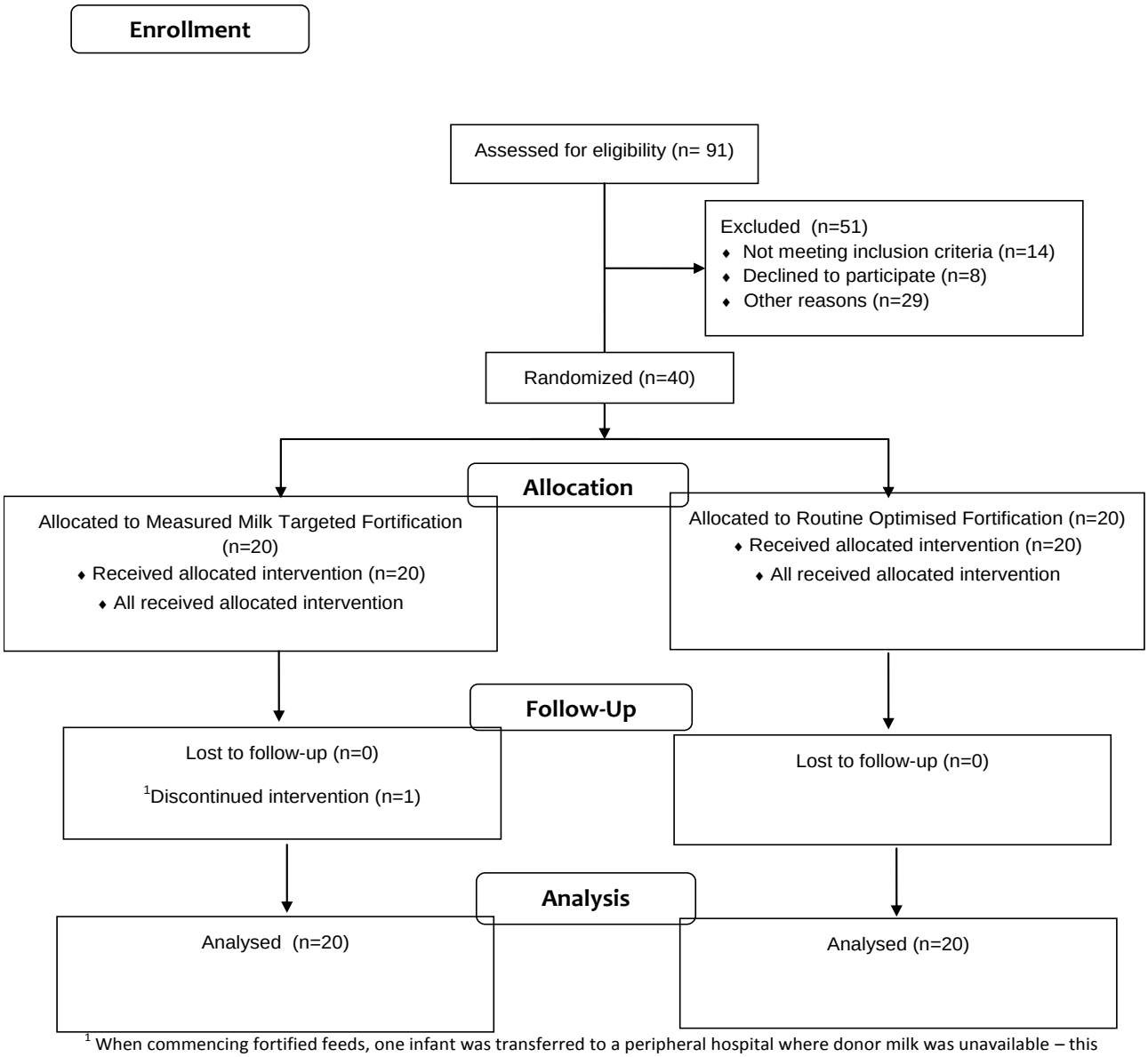
Figure 1. Enrolment



[1] When commencing fortified feeds, one infant was transferred to a peripheral hospital where donor milk was unavailable – this infant was transitioned to preterm formula and excluded only from the analysis during the fortification period.

Table 1 Protein and Energy Guidelines

Consensus Guidelines 2005[6]			**ESPGHAN Guidelines 2010[7]**	
Criteria			**Criteria**	
	Protein **PER**			**Protein** **PER**
24-30w PMA	3.8-4.4 PER 3.3-3.4		Body weight <1000 g	4.0-4.5 PER 3.6-4.1
30-36w PMA	3.4-4.2 PER 2.8-3.3		Body weight 1000 g to 1800 g	3.5-4.0 PER 3.2-3.6
36-40w PMA	2.8-3.4 PER 2.4-2.8			
	ENERGY			**ENERGY**
Birth weight <1000 g	545-629 kJ			
Birth weight 1000 g to <1500 g	461-545 kJ		Body weight 1000 g to 1800 g	461-565 kJ
All values expressed kg/d except PER (protein energy ratio – g protein per 419 kJ)				

Table 2 Clinical data of subjects

	Igp (n=20)	RPgp (n=20)	p-value
Gestational age (wk)	27.0 (1.9) 26 (23-29)	27.1 (2.0) 27 (23-29)	0.781 0.752
Birth weight (g)	1014.8 (269.3) 1022 (560-1475)	1009.2 (313.1) 1009 (480-1375)	0.953 0.752
Birth length (cm)	35.3 (3.5) 35.5 (28.5-41)	35.7 (4.6) 36.0 (27.0-42.0)	0.764 1.000
Birth head circ (cm)	25.0 (21.0-27.5) 25.0 (21.0-27.5)	25.2 (20.0-28.0) 25.2 (20.0-28.0)	0.948 1.000
Male gender	9 (45%)	10 (50%)	0.752
Apgar 1 min <7	13 (68%)	17 (85%)	0.273
Patent ductus arteriosus	13 (65%)	11 (55%)	0.519
Necrotising enterocolitis ≤ Stage 2[29]	3 (15%)	2 (10%)	1.000
Antibiotic courses ≥ 2	13 (65%)	10 (50%)	0.337
Indomethacin	8 (40%)	7 (35%)	0.744
Blood culture/s +ve	10 (50%)	7 (35%)	0.337
Blood transfusion/s	14 (70%)	11 (55%)	0.327
Recovery of birth weight (d)	10 (1-25)	10 (1-21)	0.849
Parenteral nutrition (d)	19 (6-36)	17 (6-47)	0.766
Full enteral feeds achieved (d)	17 (8-27)	17 (9-29)	0.654
Days from birth when feeds were fortified (d)	20 (10-39)	20 (10-36)	0.903
Weight at start of fortification (g)	1032 (700-1998)	1155 (505-1885)	0.925
Duration of oxygen (d) (Igp n=17, RPgp n=19)	6 (1-87)	28 (1-112)	0.163
Duration of ventilation and CPAP (d) (Igp n=19, RPgp n=20)	47 (1-89)	36 (1-95)	0.955

Data mean (SD) or median (range) or n (%)

Table 3 Macronutrient composition of milk feeds* and nutritional intakes

Composition of milk feeds (n=1870 samples)			
Per 100 mL	**Igp** n=20	**RPgp** n=20	**p-value**
Protein (g)	1.6 (0.5)	1.6 (0.1)	0.466
Fat (g)	4.3 (0.7)	4.5 (0.6)	0.332
Lactose (g)	6.8 (0.2)	6.9 (0.2)	0.133
Energy (kJ)	304 (25)	312 (22)	0.290
PER	2.3 (0.7)	2.1 (0.3)	0.237
	Calculated nutritional intakes after milk was fortified on measured composition		
	Igp: n=20	**RPgp:** n=20	
Fluid (mL)	158 (14)	153 (9)	0.256
Energy (kJ)	524 (44)	538 (47)	0.336
Protein (g)	3.3 (0.4)	3.4 (0.5)	0.673
PER	2.6 (0.3)	2.7 (0.3)	0.751
Lipid (g)	6.8 (0.9)	6.8 (1.0)	0.702
CHO (g)	12.9 (1.1)	13.5 (0.9)	0.640
	Calculated nutritional total intakes from parenteral, enteral (using measured milk composition) and IV nutrition		
	Igp: n=20	**RPgp:** n=20	
Fluid (mL)	147 (8)	146 (8)	0.555
Energy (kJ)	456 (39)	481 (48)	0.079
Protein (g)	3.2 (0.3)	3.4 (0.4)	0.067
PER	3.0 (0.5)	3.0 (0.3)	0.973
Lipid (g)	5.7 (0.9)	5.9 (0.8)	0.372
CHO (g)	11.6 (0.9)	12.3 (1.1)	0.026

Data summarised as mean (SD).
**mean macronutrient composition of milk from 14 days of an infant commencing MEF to discharge; Atwater conversion factors: protein 16 kJ/g; fat 37 kJ/g; lactose 16 kJ/g.*

Table 4 Growth data of infants at discharge.

	Igp Intervention n=20	RPgp Control n=20	p-value
	Mean (SD)		
Growth at discharge			
Age (wk)	37.7 (2.5)	37.8 (2.2)	0.762
Fat mass (g)	318 (111)	348 (149)	0.469
Body fat (%) (without correction for length)	13.7 (3.6)	13.6 (3.5)	0.984
Discharge weight (kg)	2294 (356)	2464 (528)	0.243
Discharge length (cm)	43.8 (2.6)	44.6 (2.8)	0.343
Discharge head circumference (cm)	32.4 (1.6)	33.1 (1.8)	0.184
Weight gain velocity from birth (g $kg^{-1}d^{-1}$)	11.4 (1.4)	12.1 (1.6)	0.135
Weight gain velocity after birth weight regained (g $kg^{-1}d^{-1}$)	13.4 (1.9)	14.3 (1.6)	0.139

Table 5 Modelling of Weight Gain and Body Composition with Macronutrient Intake Data

Growth Outcome	Mean effect	95% CI			p-value
Weight gain velocity*					
Intervention group	1.08	0.98	-	1.19	0.140
Enteral protein (g/kg/d)	1.09	1.01	-	1.18	0.024
Postmenstrual age	1.01	0.98	-	1.05	0.475
Postmenstrual age^2	0.99	0.98	-	0.99	<0.001
Chronological age	1.00	0.99	-	1.01	0.164
Birth weight z-score	0.92	0.86	-	0.99	0.036
Percentage fat mass at discharge					
Intervention group	0.88	-0.71	-	2.47	0.269
Female gender	3.09	1.49	-	4.69	<0.001
Postmenstrual age age	0.82	0.43	-	1.21	<0.001
Weight z-score	2.00	0.93	-	3.08	0.001
Residual (length z-score vs. weight z-score)	-1.91	-3.26	-	(-0.57)	0.007
Percentage fat mass at discharge (combined protein intake >3.4 g kg^{-1}d^{-1} vs. ≤3.4 g kg^{-1}d^{-1})					
Protein >3.4g/kg/d	-2.02	-3.98	-	(-0.05)	0.042
CHO (g/kg/d)	0.59	-0.23	-	1.41	0.153
Female gender	3.21	1.66	-	4.76	<0.001
Postmenstrual age	0.63	0.22	-	1.04	0.003
Weight z-score	1.70	0.66	-	2.74	0.002
Residual (length z-score vs. weight z-score)	-1.85	-3.15	-	(-0.54)	0.007

** Weight gain velocity was analysed using Linear Mixed Models Regression and transformed to the natural logarithm for analysis. Estimates and confidence intervals have been back transformed for this outcome in the table, with the result that each estimate now represents the proportion change in weight gain velocity. For example, an additional g/kg/day of enteral protein was associated with an average 1.09 times increase, or 9% increase in weight gain velocity (95% CI 1%-18%).*