Preterm human milk composition: a systematic literature review

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Abstract
There are wide variations in the macronutrient values adopted by neonatal intensive care units and industry to fortify milk in efforts to achieve recommended intakes for preterm infants. Contributing to this is the variation in macronutrient composition of preterm milk between and within mothers and the variable quality of milk analyses used to determine the macronutrient content of milk. We conducted a systematic review of the literature using articles published in English between 1959 and 2013 that reported the concentrations of one or more macronutrients or energy content in human preterm milk, sampled over a representative 24-h period. Searchmedicall databases included Ovid Medline, Scopus, CINAHL and the Cochrane Library. Results are presented as mean values and ranges for each macronutrient during weeks 1–8 of lactation, and preferred mean values (g/100 ml) for colostrum (week 1) and mature milk (weeks 2–8; protein: 1·27, fat: 3·46, lactose: 6·15 and carbohydrate: 7·34), using data from studies employing the highest-quality analyses. Industry-directed fortification practices using these mean values fail to meet protein targets for infants weighing <1000 g when the fortified milk is fed <170–190 ml/kg per d, and the protein:energy ratio of the fortified milk is inadequate. This study aimed to provide additional information to industry in order to guide their future formulation of breast milk fortifiers. Quality macronutrient analyses of adequately sampled preterm breast milk would improve our understanding of the level of fortification needed to meet recommended protein and energy intakes and growth targets, as well as support standardised reporting of nutritional outcomes.

Key words: Preterm infants; Breast milk; Composition; Macronutrients; Fortification

Compared with healthy term infants, preterm infants are born physiologically immature, nutritionally compromised, growth restricted and at risk of long-term complications¹⁻³⁰. The optimal nutrition for preterm infants is to be fed human breast milk¹⁻³⁵, and to mirror the growth and development of the age-matched healthy fetus. However, the nutrient and energy contents of native breast milk are insufficient to meet the needs of the preterm infant¹⁻³⁵, and fortification processes are thus used to assist in achieving the latest European recommended intakes⁴⁰. These recommendations target 460–565 kJ/kg per d (110–135 kcal/kg per d), with 4·0–4·5 g protein/kg per d (3·5–4·1 g protein/418·4 kJ (100 kcal)) for preterm infants weighing <1000 g and 3·5–4·0 g protein/kg per d (3·2–3·6 g protein/418·4kJ (100 kcal)) for infants weighing between 1000 and 1800 g⁴⁵.

Common practice is to fortify on an assumed average preterm breast milk composition⁶⁻⁷,⁹; however, significant variation in the macronutrient content of breast milk exists between and within mothers⁶⁻¹⁰,²², reflected by wide variations in macronutrient values derived by studies and adopted by neonatal intensive care units and industry to fortify milk, raising concerns that this practice may lead to undernourishment of some infants and over-nourishment of others⁶⁹. The lack of a globally accepted reference ‘assumed’ preterm breast milk composition inhibits standardised reporting of nutritional intakes, making it difficult for clinicians and researchers to assess the adequacy of nutritional intakes and the role of nutrition in growth and developmental preterm outcomes.

Macronutrient composition, particularly lipid concentration, is dependent on the method of sampling, stage of lactation, gestational age, maternal diet, presence of maternal infection and parity¹³⁻²³. In addition, there is significant diurnal and inter-feed variation; as such, study designs that incorporate milk expressions collected at each feed over a 24-h period are preferred to ensure that analysis is being undertaken on a representative milk sample²⁴. Accurately measuring the composition of individual breast milk feeds is difficult, costly and time consuming, and studies have collectively differed in the quality of their various methodologies and analytical designs.

Abbreviations: BMF, breast milk fortifiers; EBM, expressed breast milk; NPN, non-protein nitrogen; TN, total nitrogen; PER, protein:energy ratio; PN, protein nitrogen.

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Bedside milk analysis with the potential for targeted fortification is under development, with a recent study evaluating the accuracy of using IR analysis for small aliquots of milk, clinically relevant due to the smaller volumes of milk sometimes expressed by preterm mothers. Fusch et al. highlighted that the near- and mid-IR spectroscopy equipment had variable accuracy when calibrated against micro analyses methods (0.2 g/100 ml for protein, 0.5 g/100 ml for lipid and inaccurate measurements of lactose), which can be significant when feeding at low volumes. These values were similarly found in another study reviewing mid-IR spectroscopy, with lactose again being over-estimated. A recent meta-analysis reported the mean values for each macronutrient in preterm and term milk from healthy mothers during the first 12 weeks of lactation, based on all available data, independent of the analysis used. Inclusion criteria stipulated 24-h milk collections for fat and energy only. To account for study differences in the estimation of protein in breast milk (either including or excluding non-protein nitrogen (NPN)), the authors conducted two meta-analyses of protein using the available data on each: an estimate of protein based on NPN, the authors conducted two meta-analyses of protein estimation methods in each of the studies, suggest preferred reference values for fortification based on this assessment and to determine the adequacy of industry-directed routine fortification in meeting nutrition targets.

Methods

Ovid Medline, Scopus, CINAHL and the Cochrane Library were searched using the strategy outlined in Fig. 1, limited to English language and publications from 1959 to 2013. Grey literature was identified through Trove limited to theses, ProQuest Dissertations and Theses and OAIster using the same search string.

The article review and exclusion processes are outlined in Fig. 2. In summary, all retrieved articles (n 7731) were reviewed by title and abstract, excluding articles identified as duplicates, non-English or not relevant to the study aim (Stage 2, Fig. 2). This was achieved by distributing all articles among the first four authors, who independently assessed the articles, conferring only when there was ambiguity about exclusion criteria. The remaining articles were reviewed at Stage 3 (Fig. 2) by at least two authors, and further exclusions were made if full texts could not be sourced, or if studies did not analyse preterm human milk for protein, energy, carbohydrate or lipid contents in representative 24-h samples. A 24-h sampling procedure was deemed necessary in order to obtain representative samples that accounted for diurnal and inter-feed variation in composition. We included 24-h milk expressions or representative samples comprising fore- and hind-milk samples collected in equal volumes from each expression throughout the day. Articles were also critiqued according to sample size, number of milk samples analysed and maternal demographics.

The included articles were examined with the aid of a hierarchical ranking of analytical methods (Fig. 3) based on the EN ISO/IEC 17025:2005 methodology practice, which considers methods according to selectivity, measurement uncertainty, repeatability/reproducibility and robustness within the matrix. A method ranking system was not necessary for energy as the primary methods of determination were consistent within the articles assessed. Analyses expressing total carbohydrate and those only expressing the disaccharide lactose were considered separately.

All values were collated and standardised to g/100 ml for each macronutrient and kJ (kcal)/100 ml for energy and were
assigned to a lactation week according to the day of lactation (i.e. lactation week 1 = inclusive of 1–7 d, lactation week 2 = inclusive of 8–14 d, etc.) with values >56 d compiled and reported as ≥ week 8. Values that spanned multiple weeks were assigned to a week by the median day of the time period reported. In the case of values reported by type of milk, these were deemed to be of weeks 1, 2 and 4 for colostrum, transitional and mature milk, respectively.

Protein as determined by the Kjeldahl method results in determination of TN, requiring adjustment for NPN and the subsequent conversion of this derived value to protein equivalents(29). In this review, protein is defined as bioavailable N multiplied by the general conversion factor 6·25(30). Bioavailable N is protein nitrogen (PN = TN – NPN) plus the proportion of NPN that is available for protein synthesis (approximately 27% of the NPN)(31).

NPN values specific to different weeks of lactation (17·6% for weeks 1–4 of lactation(21); 24% after week 4(31)) were used to standardise protein values across studies and to ensure that all values were representative of bioavailable proteinaceous material. To achieve this, all reported values for PN were back calculated to TN. All TN values were then converted into bioavailable N using the applicable assumed NPN percentage. In addition, where studies reported PN derived from the chemical determination of NPN, adjustment was made for bioavailable N where necessary. As there was little difference between the calculated and the chemically derived NPN component of milk, all studies using a form of the Kjeldahl method were used to suggest preferred mean protein values for weeks 1–8 of lactation.

Results

In all, 7731 articles were reviewed by title and abstract, and 7039 articles were excluded as they were duplicates, non-English or not relevant to the study aim (Stage 2, Fig. 2). Of the 692 articles reviewed at Stage 3 (Fig. 2), a further 668 articles were excluded because the investigators did not analyse preterm human milk for protein, energy, lactose, carbohydrate or lipid contents in representative 24-h samples or because full texts could not be sourced. The demographics of the included studies are reported in Table 1.

Results from the twenty-four studies included in this review are collated in Table 2 and depict mean and standardised reference ranges, organised by lactation week and macronutrients. To determine a more accurate macronutrient composition, the means/medians (minimum, maximum) of the mean values reported in lactation week 1 and weeks 2–8 by studies using the more robust methodology as per Fig. 3 are shown in Table 3. Protein values used contain bioavailable protein based on assumed NPN percentage. Energy values have been calculated from the mean values using the Atwater general factors(49). The mean and minimum, maximum values reported for lactation weeks 2–8 (Table 3) have been used to calculate incremental volume intakes (ml/kg per d) of breast milk that has been routinely fortified with human milk fortifier (Nutriprem, Cow and Gate; Nutricia). The fortifier provides an additional 1·1 g of protein, 2·8 g of carbohydrate and 67 kJ (16 kcal) of energy when added to 100 ml of expressed breast milk (EBM) (Fig. 4 (a–c)). Table 4 illustrates the change to protein:energy ratio (PER) and quantifies protein intakes at incremental volumes
Discussion

Biological outcomes

Individualising milk fortification on the basis of measured milk analysis to meet recommended intakes and growth targets is not always possible or pragmatic in the clinical setting. It requires consideration of the infant’s clinical presentation, weight and prescribed feeding volume, is reliant on precise, accurate and expensive measuring equipment, and is labour intensive and time consuming. Instead, and commonly, fortifier is added to milk in routine amounts as directed by industry. The calculation and reporting of nutritional intakes is thus based on the formulation of the fortifier and an assumed milk composition. There are wide variations in milk composition between and within mothers and across the course of lactation, and there are many variants in the assumed milk composition used across neonatal centres to estimate intakes. Therefore, it is difficult to interpret studies that seek to investigate the role nutrition plays in clinical and growth outcomes in the neonatal setting, which ultimately leads to a paucity of good-quality, standardised data that can inform both nutritional practice and development of evidence-based nutritional guidelines.

In this review, we have reported the mean values and ranges of the macronutrient content of preterm breast milk per lactation week, using data from studies that used 24-h milk sampling (Table 2). This strategy was adopted to avoid making broad assumptions or oversimplifying data that are influenced by differences in study design and by the diurnal, within-feed and inter- and intra-maternal variations in milk composition. Selecting data from studies that have utilised the most robust methodology, we have suggested preferred reference mean values for the macronutrient composition of preterm colostrum and preterm mature breast milk (protein: 1·27; fat: 3·46; carbohydrate: 7·34; energy: 66) for use in the clinical setting (Table 3). Global acceptance of these reference values may help standardise calculation and reporting of nutritional intakes and the development of evidence-based guidelines and sound nutritional practice. This will better direct clinicians, researchers and industry in the development of appropriate formulations of breast milk fortifiers (BMF) that address the wide variation in breast milk composition and guide the level of fortification required to better achieve preterm nutrition and growth targets.

Growth data of 13 years (2000–2013) collected from 362833 low birth weight infants (501–1500 g) in the Vermont Oxford Network(50) have recently been published. These growth data were collected over the period when earlier and more aggressive nutrition regimens were being adopted and during a time when the highest-ever protein intakes were recommended for infants weighing <1000 g(8,51). It is concerning that as late as 2013, 50% of low birth weight infants in this network were growth restricted at discharge (<10th percentile), and 28% had severe growth failure (<3rd percentile)(50). This is despite the reformulation of several human milk fortifiers since the latest enteral guidelines were released in 2010(8), suggesting that current nutrition regimens, including fortification practices, may still not be sufficiently optimised to meet the needs of extremely preterm infants.
### Table 2. Standardised protein, lipid, lactose/carbohydrate and energy values organised by lactation week

(Standardised mean values and ranges)

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Author</th>
<th>Protein method based on</th>
<th>Protein value (g/100 ml)</th>
<th>Lipid method based on</th>
<th>Lipid value (g/100 ml)</th>
<th>Lactose/carbohydrate value (g/100 ml)</th>
<th>Energy value (kJ/100 ml)</th>
<th>Energy value (kcal/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anderson(18)<em>, Anderson(18)</em>, Atkinson(16)<em>, Atkinson(16)</em>, Atkinson(16)<em>, Atkinson(16)</em>, Beiers(94)<em>, Beiers(94)</em>, Guerney(90)<em>, Guerney(90)</em>, Jitta(95)<em>, Lemons(91)</em>, Lucas(93)<em>, Sann(46)</em>, Stevens(16)*</td>
<td>Kjeldahl(16,34), Kjeldahl without NPN, correction(18,20,35,46,48), Micro Kjeldahl(17), Semi-micro Kjeldahl without NPN correction(36,43)</td>
<td>1.99 1.71–2.60</td>
<td>Folch(37,46), Roese–Gottlieb(30,45), Colorimetry(16,18,33), De la Huerga(36), Van de Kamez(17), Creamatocrit(44), IR spectrophotometry(38)</td>
<td>2.80 1.60–3.90</td>
<td>Lactose: Chromatography(16,34), Boehringer lactose assay kcf(35,45), Unidentified(35,44)</td>
<td>6.01 5.04–7.12</td>
<td>4.62 3.98–5.26</td>
</tr>
<tr>
<td>2</td>
<td>Anderson(18)<em>, Atkinson(16)</em>, Atkinson(16)<em>, Atkinson(16)</em>, Atkinson(16)<em>, Beiers(94)</em>, Beiers(94)<em>, Corvaglia(13), Ehrenkrantz(13), Gross(13), Guerney(90)</em>, Jitta(95)<em>, Lemons(91)</em>, Lepage(92)<em>, Lucas(93)</em>, Maas(94)<em>, Sann(46)</em>, Stevens(16)*</td>
<td>Kjeldahl(16,34,43), Kjeldahl with NPN not accounted for, correction(18,20,35,33,47,48), Micro Kjeldahl(17), Semi-micro Kjeldahl without NPN correction(36,43)</td>
<td>1.67 1.46–2.40</td>
<td>Folch(37,46), Roese–Gottlieb(30,45), Colorimetry(16,18,33), De la Huerga(36), Van de Kamez(17), Creamatocrit(44), IR spectrophotometry(13)</td>
<td>3.63 3.49–4.30</td>
<td>Lactose: Chromatography(16,34), Boehringer lactose assay kcf(35,45), Unidentified(35,44)</td>
<td>5.94 5.31–6.86</td>
<td>4.49 3.94–5.46</td>
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<td>3</td>
<td>Anderson(18)<em>, Atkinson(16)</em>, Atkinson(16)<em>, Atkinson(16)</em>, Atkinson(16)<em>, Beiers(94)</em>, Beiers(94)<em>, Ehrenkrantz(13), Gross(13), Jitta(95)</em>, Lemons(91)<em>, Lucas(93)</em>, Maas(94)<em>, Sann(46)</em>, Stevens(16)*</td>
<td>Kjeldahl(16,34), Kjeldahl without NPN correction(18,20,35,44,45,46), Semi-micro Kjeldahl without NPN correction(42,43)</td>
<td>1.37 0.88–2.10</td>
<td>Folch(37), Roese–Gottlieb(44), Colorimetry(16,18,33), Creamatocrit(44)</td>
<td>3.84 3.24–4.80</td>
<td>Lactose: Chromatography(16,34), Boehringer lactose assay kcf(35,45), Unidentified(35,44)</td>
<td>6.07 5.76–7.50</td>
<td>4.72 4.36–5.42</td>
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<td>4</td>
<td>Bauer(16)<em>, Faer(20)</em>, Guerney(90)<em>, Lemons(91)</em>, Maas(94)*</td>
<td>Kjeldahl(11), Kjeldahl without NPN correction(44), Lowry–Peterson(48)</td>
<td>1.33 0.93–2.00</td>
<td>Folch(37), Roese–Gottlieb(44), De la Huerga(36), IR spectrophotometry(38)</td>
<td>3.88 3.50–4.60</td>
<td>Lactose: Chromatography(16,34), Boehringer lactose assay kcf(35,45), Unidentified(35,44)</td>
<td>6.02 7.40–7.53</td>
<td>4.77 4.36–5.42</td>
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<td>5</td>
<td>Bauer(16)<em>, Ehrenkrantz(27), Lemons(91)</em>, Maas(94)*</td>
<td>Kjeldahl(10), Kjeldahl without NPN correction(44), Lowry–Peterson(48)</td>
<td>1.46 1.23–1.90</td>
<td>Folch(37), Roese–Gottlieb(44), Colorimetry(110)</td>
<td>3.63 3.17–4.36</td>
<td>Lactose: Chromatography(16,34), Boehringer lactose assay kcf(35,45), Unidentified(35,44)</td>
<td>6.32 7.71–7.21</td>
<td>5.01 4.36–5.42</td>
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</table>

Note: The values are mean and range, and they are subject to the Cambridge Core terms of use, available at [www.cambridge.org/core/terms](https://www.cambridge.org/core/terms).
### Table 2. Continued

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Author</th>
<th>Protein method based on</th>
<th>Protein value (g/100 ml) Mean</th>
<th>Range</th>
<th>Lipid method based on</th>
<th>Lipid value (g/100 ml) Mean</th>
<th>Range</th>
<th>Lactose/carbohydrate value (g/100 ml) Mean</th>
<th>Range</th>
<th>Energy method based on</th>
<th>Energy value (kJ/100 ml) Mean</th>
<th>Range</th>
<th>Energy value (kcal/100 ml) Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Bauer(6)*, Faer(38), Maas(44)†</td>
<td>Kjeldahl without NPN correction(14)</td>
<td>1.38</td>
<td>1.05–1.90</td>
<td>Roese–Gottlieb(44) IR spectrophotometry(38)</td>
<td>4.24</td>
<td>3.57–4.90</td>
<td>Lactose: Boehringer lactose assay kit(44)</td>
<td>6.18</td>
<td>7.30–7.50</td>
<td>Heats of combustion factors(16,44)</td>
<td>331.4</td>
<td>293.3–362.3</td>
<td>79.2</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td>Total carbohydrate: Carbohydrate by difference(44) IR spectrophotometry(38)</td>
<td>7.40</td>
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<td>Bomb calorimetry(16)</td>
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<td>7</td>
<td>Bauer(44)†, Faer(38), Lemons(16)†, Maas(44)†, Saarela(45)</td>
<td>Kjeldahl(14)</td>
<td>1.31</td>
<td>1.03–1.80</td>
<td>Roese–Gottlieb(44,45) Colorimetry(16) IR spectrophotometry(38)</td>
<td>3.92</td>
<td>2.94–4.80‡</td>
<td>Lactose: Chromatography(16) Boehringer lactose assay kit(44)</td>
<td>6.83</td>
<td>5.95–7.45‡</td>
<td>Atwater general factor system(45)</td>
<td>290.8</td>
<td>263.6–334.7</td>
<td>69.5</td>
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<td>≥8</td>
<td>Anderson(116)*, Atkinson(118)†, Bauer(44)†, Bejons(142)†, Ehrenkranz(37), Gross(147)†, Jitter(46), Lemon(16)†, Lucas(144)†, Maas(44)†, Saarela(45) STEVENS(148)</td>
<td>Kjeldahl(14,34)</td>
<td>1.37</td>
<td>0.88–2.10</td>
<td>Folch(37) Roese–Gottlieb(20,44,45) Colorimetry(16,18) Creamatocrit(16,40)</td>
<td>3.84</td>
<td>3.24–4.80</td>
<td>Lactose: Chromatography(16) Boehringer lactose assay kit(16,44)</td>
<td>6.07</td>
<td>5.76–7.50</td>
<td>Atwater specific factor system(45)</td>
<td>297.9</td>
<td>252.3–323.8</td>
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<td>Total carbohydrate: Carbohydrate by difference(44) Colorimetry(16)</td>
<td>7.42</td>
<td>7.25–7.60</td>
<td>Bomb calorimetry(16,45)</td>
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</table>

NPN, non-protein nitrogen.
* Articles from which one or more tabulated values were extracted from graphs.
† Articles in which protein values were calculated from reported N values, using the conversion factor 6.25.
‡ Ranges inclusive of values obtained up to 16 weeks of lactation.

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Commercial BMF currently provide between 1·0 and 1·1 g protein/100 ml EBM. Polberger et al. (52) used a BMF containing 1·0 g protein/100 ml EBM to routinely fortify milk and calculated protein intakes of 3·05 g/kg per d in the control arm of the trial, using milk composition data measured with IR technology. Similarly, McLeod et al. (53) used a BMF containing 1·0 g protein/100 ml EBM and additional protein powder up to 0·5 g/100 ml EBM to fortify the milk of preterm infants in the control arm of a randomised trial. Mean volume and protein intakes of 153·5 ml/kg per d and 3·9 g/kg per d, respectively, were calculated for these infants during the fortification period, using milk composition data obtained with mid-IR technology. Miller et al. (54) has also shown improved protein intakes of 4·2 g/kg per d in the first 4 weeks of a study of preterm infants born <31 weeks of gestation who were fed a trial-based BMF containing 1·4 g of protein/100 ml of breast milk (160, 139–170 ml/kg per d) compared with controls who were fed an isonitrogenic BMF containing 1·0 g protein to achieve intakes of 3·6 g/kg per d (164, 149–171 ml/kg per d).

The American Academy of Pediatrics has stipulated that breast milk should be the primary diet of all preterm infants and that it should be appropriately fortified for those with birth weights <1500 g to target intra-uterine growth rates (55). Using our preferred minimum and mean values for preterm milk (Table 3) and by using fortification levels as directed by industry, we have demonstrated (Fig. 4(c)) that the recommended protein targets of 4·0–4·5 g/kg per d considered necessary for the growth of preterm infants weighing <1000 g (60) may not be achieved below volumes of 190–210 and 170–190 ml/kg per d, respectively. This is concerning, given that lower volumes may be prescribed for extremely preterm infants to minimise risk of long-term morbidity such as chronic lung disease and patent ductus arteriosus (60). It is also concerning that the PER of fortified milk, calculated using these preferred values, does not appear to meet recommendations for these infants (Fig. 4(e)). From our estimations, it seems likely that this is due to the insufficient protein and high carbohydrate content of fortifiers, the latter adding a substantial energy load to the final composition of fortified milk, resulting in excessive energy intakes at reasonably low volumes (Fig. 4(d)) and carbohydrate intakes exceeding recommendations beyond volumes of 140 ml/kg per d (Fig. 4(b)).

Table 4 depicts simulated fortified intakes achieved at incremental fluid intakes, based on our preferred mean values, and a commercially available BMF (1·1 g protein/100 ml EBM) and 0·5 or 1·0 g of protein supplement (0·86 g protein/g protein powder). Notably, protein, energy and PER targets for infants weighing <1000 g are achieved when 0·5 g of protein powder is added to feeds in addition to BMF and fed at volumes between 140 and 160, and similarly these targets are achieved at lower volumes (130–140 ml/kg per d) when 1·0 g of protein powder is added in addition to routine amounts of BMF. This level of fortification is currently being practised in some neonatal units around the globe, with the understanding that additional, intact protein powder does not adversely affect osmolality of the feeds (55), and with the acceptance that protein intakes may be greater than needed for some preterm infants if the native content of the milk is actually higher than anticipated (60). The efficacy and safety of these fortification practices have not been well studied, and further research is needed to determine the best formulation and the amount of fortifier that can be safely added to preterm milk to optimise preterm growth, developmental and metabolic outcomes. It seems increasingly clear that one size may not fit all; different strength fortifiers may be required to meet the needs of very low weight or poorly growing infants and to address the variations in milk composition between mothers and across the course of lactation.

### Justification for the preferred reference values of preterm milk

**Protein.** Amino acid analysis is the most accurate method for determining true protein content (22); however, this method is time consuming and costly. (22) The Kjeldahl method is the most accurate indirect determination of protein content (57, 58) and it – or its more sensitive derivations (59) (micro and semi-micro Kjeldahl) – is the most commonly used method employed by studies in this review (13, 16–18, 20, 21, 32–34, 42–45, 47, 48). The Kjeldahl method involves liberation of TN from a sample (60). A second analysis improves the accuracy by determining NPN and subtracting this from TN to determine PN (60). During protein precipitation, peptides remain in the supernatant and are attributed to NPN, resulting in a minor underestimation of true protein (60). Justification for these assumptions follows. The Kjeldahl method is currently being practised in some neonatal units around the globe, with the understanding that additional, intact protein powder does not adversely affect osmolality of the feeds (55), and with the acceptance that protein intakes may be greater than needed for some preterm infants if the native content of the milk is actually higher than anticipated (60). The efficacy and safety of these fortification practices have not been well studied, and further research is needed to determine the best formulation and the amount of fortifier that can be safely added to preterm milk to optimise preterm growth, developmental and metabolic outcomes. It seems increasingly clear that one size may not fit all; different strength fortifiers may be required to meet the needs of very low weight or poorly growing infants and to address the variations in milk composition between mothers and across the course of lactation.
The modified Lowry–Petersen and Biuret assays use colorimetry to quantify protein concentration\(^{(60)}\). The Biuret assay, used by Sann et al.\(^{(46)}\), is limited in that it requires a large number of samples\(^{(61)}\), has low sensitivity with detection starting at 1 g/l and can be confounded by other components in milk\(^{(60)}\). Bauer & Gerss\(^{(6)}\) used the Lowry–Petersen method to determine protein content in the milk of a large sample of mothers; this study reported the highest protein values for each lactation week. Reliability of the Lowry–Petersen assay is dependent on technique, which demands high accuracy in
Achieving a 100-fold dilution and precise timing when adding the reagent.\(^{60}\)

Jitta et al.\(^{40}\) used a variation of the Esbach's method, which was designed to measure protein in urine.\(^{62}\) This, together with the socio-cultural demographics of the Kenyan women in this study\(^{40}\), may account for the low protein values reported for weeks 3 and 4 compared with those determined by other methods.

In addition, two studies used IR spectrophotometry methods to determine TN and true protein concentrations, respectively\(^{13,38}\). This indirect determination can vary in accuracy depending on the calibration of the individual machines\(^{12}\), the choice of the reference method used and the diversity and number of samples used in the calibration set\(^{63}\). That aside, it is noteworthy that the protein values reported in these studies were consistent with values found by more direct methods.

Lipid. During the first 5 weeks of lactation, lipid concentration appears to increase, with convergence of the range of reported values. Obtaining representative lipid samples requires 24-h collections to allow for variation as well as careful mixing of the sample at 38°C before removing an aliquot for analysis\(^{43}\). The preparation and storage of a sample is important to maintain its integrity; a limit of 14 d at −20°C is recommended to prevent lipolysis\(^{64}\). Only one study specified the duration of storage at such temperatures\(^{45}\). This susceptibility to lipolysis can be overcome by storing samples at −70°C or below\(^{64}\), a strategy used in three of the included studies\(^{6,16,37}\). Of note, this should be used by studies determining lipid concentration without using extraction techniques. Preferred methods for determining total lipid concentration include modified Folch and Roese–Gottlieb methods, which both use chloroform–methanol extraction followed by gravimetric determination\(^{64,65}\). Insull & Ahrens\(^{60}\) compared modified Folch with Roese–Gottlieb methods, and found that the total lipids that were recovered were similar between the two methods but the Folch method recovered phospholipids more effectively\(^{25}\).

Two studies used IR spectrophotometry methods in these studies emphasises the robust nature of their methodology. The Folch technique was used by Ehrenkranz et al.\(^{37}\) and Sann et al.\(^{40}\); however, the reported results using this method were only comparable for matched lactation weeks 1 and 2, in which differences in fat concentration of up to 1.35 g/100 ml were noted.

Chloroform–methanol extraction followed by colorimetric determination was used by Anderson et al.\(^{18}\) and Atkinson et al.\(^{35}\). Lemons et al.\(^{16}\) used colorimetric determination but used sulphuric acid for extraction\(^{67}\). The colorimetric determination of lipid yielded higher values for week 1 of lactation, compared with those determined by the Roese–Gottlieb and Folch methods, but were similar for the remaining weeks. A modified De la Huerga method of extraction followed by photometric determination was used by Guerrini et al.\(^{39}\). This method was not as specific as previously mentioned methods, as there was no precipitation of protein before achieving a 100-fold dilution and precise timing when adding the reagent\(^{60}\).
photometrically reading the sample’s turbidity. The method used by Silber et al.(47) was crude fat determination as lipid extraction was performed using non-polar solvents, and therefore did not account for phospholipids. Anderson et al.(17) calculated total lipid concentration on the basis of average fatty acid chain length, a method less robust than direct measurements.

The creamatocrit technique is a simple, rapid and inexpensive measure of fat in milk(64,68). The method is limited by the subjectivity associated with reading the measurement, and the potential for overestimating the lipid content due to significant unpacking of the lipid column if the sample is read after 30 min(6,68). However, the creamatocrit method has an accuracy of ±10%, which is considered by some to be adequate in most clinical and research settings(68). Jitta et al.(40) and Bauer & Gens(6) both used the creamatocrit technique, and their results were comparable with the Roese–Gottlieb method for weeks 1–3 but appeared to be an overestimation for week 4. The Gerber method used by Corvaglia et al.(13) uses an adequate extraction technique; however, the volumetric determination is inferior to the gravimetric methods and requires a large sample size(6).

IR spectrophotometers have the ability to ascertain values for all macronutrients in one measurement; however, this is an indirect measurement and the machine must be calibrated against direct analysis(65). The strength of the calibration depends on the reference chemistry used and the number and range of samples(65). Homogenisation is an important step in preparing milk samples as it decreases the variability in fat globule size and subsequent light-scattering effect of larger globules, improving the accuracy of measurement(29). Reducing the diameter of the fat globule to <5μm can be achieved through either manual or ultrasonographic methods; however, utilising an ultrasound processor is associated with high measured values for N, energy and particularly fat and lactose(69). Neither of the studies has reported whether the samples underwent homogenisation before analysis. Corvaglia et al.(13) used near-IR, with a detailed description of calibration. This study found a correlation of 0.808 between IR analysis and the Roese–Gottlieb method(13). Faerk et al.(38) used mid-IR spectrophotometry but did not provide information on the calibration of their machine, which is critical in establishing the reliability of the results(65). This study consistently reported higher lipid concentrations than those reported by others using different methods.

**Carbohydrate/lactose.** Lactose is the predominant disaccharide carbohydrate in human milk (approximately 70–85% of total carbohydrates); however, human milk also contains free glucose and galactose, as well as numerous oligosaccharides(70). Of the studies reported in Table 1, nine reported lactose concentrations(16,18,20,33,40,44–47) and five reported total carbohydrate(6,17,30,36,44). As expected, the total carbohydrate composition range was consistently higher than lactose. Further, both lactose and total carbohydrate followed the same trend, which was to increase gradually over weeks 1–4 and remain relatively stable from then on. Although it may be preferable from a clinical perspective to identify a total carbohydrate value, the majority of studies with high methodological quality measured lactose; two reliable methodologies identified for lactose determination were used by five studies(16,30,33,44,46). These methods were chromatography, with Lemons et al.(16) using GLC and Sann et al.(46) using ion-exchange chromatography, as well as the Boehringer lactose assay used by Anderson et al.(18), Maas et al.(44) and Atkinson et al.(53). Although these methods differ, they are each primary methods of analysis, specific for lactose and are not confounded by carbohydrate interference. The chromatographic methods achieve this by virtue of their calibrated parameters being lactose specific, and the Boehringer lactose assay accounts for free glucose. There were four studies included in this review reporting lactose values in which the methodology was unable to be sufficiently identified(20,40,45,47). It is also noteworthy that Saarela et al.(45) used an unidentified enzymatic degradation method, which greatly increased the range reported in weeks 1 and 4 where values from their study were included. It is possible that this methodology may not have accounted for free glucose, and thus overestimated lactose; however, this is unlikely to be significant. Of the studies reporting total carbohydrate, the main limitation was the infrequency with which measurements were taken or reported over the weeks of lactation. Chessex et al.(50) and Bauer & Gens(6) only contributed to the ranges reported in weeks 3 and 4, respectively. Although Faerk et al.(38) reported values for multiple weeks, it should be noted that the validity of the IR spectrophotometry values is not clearly elucidated because the calibration method was not explicitly stated. Despite the potential for confounding errors in a multistep process, Maas et al.(44) used sound methodology, calculating total carbohydrate by difference, and provided the greatest contribution to the carbohydrate trend, reporting values over weeks 2–8.

**Energy.** Bomb calorimetry accurately measures total energy content and was used in a number of studies(6,16,17,42–47), however, it does not differentiate between gross and metabolisable energy, which is dependent on the bioavailability of each macronutrient in the food source. This difference can result in an overestimation of energy based on inaccuracies in each macronutrient and carbohydrate with the factors 17, 38 and 17 kJ/g (4, 9 and 95 kcal/g) for protein, fat and lactose, respectively, which are based on heats of combustion and comparable with bomb calorimetry values(18,71). Saarela et al.(45) used the Atwater general factor system, which represents the metabolisable energy of protein, fat and carbohydrate with the factors 17, 38 and 17 kJ/g (4, 9 and 4 kcal/g), respectively(36,72). These factors were further developed to be food specific, forming the Atwater specific factor system(30). This system was used by Gross et al.(20) with the factors for metabolisable energy in milk being 17.86, 36.78 and 16.19 kcal/g (4.27, 8.79 and 3.87 kcal/g) for protein, fat and...
carbohydrate, respectively. Many authors have used lactose concentration in place of carbohydrate when calculating energy content, because of lactose being the primary carbohydrate in milk, and a better reflection of the carbohydrate component that is digestible, with the remaining component being largely indigestible oligosaccharides. Although the Atwater factor systems are clinically relevant methods of energy determination, there is also the larger measurement error to consider when using factors, as the percentage errors involved in the measurement of individual components of the milk combine to give a much larger error for the derived energy content. As expected, reported energy values based on digestible or metabolisable energy factors were consistently lower than those based on total energy intake values.

Limitations of this review

Although the authors made every effort to procure papers, a small number of articles that could not be excluded by title or abstract could not be obtained for use in this review. Chemical analyses used in some studies were incompletely described or cited inaccessible reference material; these studies were mentioned within the discussion; however, the validity of the results could not be appreciated. The graphical representation of data without numerical support was a barrier to accurate interpretation. Rather than exclude these studies, the four reviewers independently extracted values manually and reached a consensus value for each relevant weekly average. However, there were many fundamental differences between studies, including number of mothers included, various maternal factors (including age, parity, nutritional and socioeconomic status), gestational ages of the infants and methodologies used, which could not be controlled for and may have contributed to the large range of values identified in the literature.

Conclusion

This review has provided a compilation of the published values of preterm human milk composition, and has suggested preferred reference values for the assumed macronutrient composition of preterm milk for week 1 (colostrum) and weeks 2–8 of lactation (mature milk). We have calculated estimated macronutrient and energy intakes of infants at prescribed fluid volumes using the minimum, mean and maximum reference values for mature milk and routine fortification practices, as well as demonstrated that recommended protein targets are likely unachievable below volume intakes of 170–190 ml/kg per d when the milk has low and average protein content. We have also demonstrated that these fortified preterm milk feeds are unlikely to have an adequate PER, potentially compromising adequate growth. Given the variable composition of breast milk and the fact that preterm infants are a heterogeneous population, different strength fortifiers may be required to meet the needs of very low weight or poorly growing infants. Global acceptance of the recommended preferred reference values for preterm milk composition may ensure standardised calculation and reporting of nutritional intakes, better direct clinicians, researchers and industry in the development of appropriate formulations of BMF, and guide the level of fortification required to better achieve preterm nutrition and growth targets.

None of the studies included in this review used the most accurate methods for measuring each macronutrient in preterm human milk. In order to determine the most accurate preterm breast milk composition, analyses must be undertaken of all macronutrients in 24-h collections from a large sample of healthy mothers over the course of lactation, using the best methodologies available.

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