

Title Page**Breast milk nutrient content and infancy growth****Authors & affiliations:**

Dr Philippa Prentice, Department of Paediatrics, University of Cambridge MRC Wellcome Trust-MRC Institute of Metabolic Science, NIHR Cambridge Comprehensive Biomedical Research Centre, UK

Dr Ken K Ong, Department of Paediatrics, University of Cambridge MRC Wellcome Trust-MRC Institute of Metabolic Science, NIHR Cambridge Comprehensive Biomedical Research Centre, UK, MRC Epidemiology Unit, University of Cambridge, UK

Dr Marieke H Schoemaker, Mead Johnson Pediatric Nutrition Institute, Nijmegen, Netherlands

Dr Eric AF van Tol Mead Johnson Pediatric Nutrition Institute, Nijmegen, Netherlands

Professor Jacques Vervoort Wageningen University, Netherlands

Professor Ieuan A Hughes, University of Cambridge MRC Wellcome Trust-MRC Institute of Metabolic Science, NIHR Cambridge Comprehensive Biomedical Research Centre, UK

Dr Carlo L Acerini Department of Paediatrics, University of Cambridge MRC Wellcome Trust-MRC Institute of Metabolic Science, NIHR Cambridge Comprehensive Biomedical Research Centre, UK

Professor David B Dunger Department of Paediatrics, University of Cambridge MRC Wellcome Trust-MRC Institute of Metabolic Science, NIHR Cambridge Comprehensive Biomedical Research Centre, UK

Short title: Breast milk and infancy growth

Corresponding author: Professor David Dunger, Box 116, Addenbrooke's Hospital, Hills Road, Cambridge CB2 0QQ

Tel: +44 1223 336886

Fax: +44 1223 336996

Email: dbd25@cam.ac.uk

Statement of financial support: PP was supported by a MRC Clinical Training Fellowship (G1001995). The Cambridge Baby Growth Study has been supported by the European Union, the World Cancer Research Foundation International, the Medical Research Council, the NIHR Cambridge Comprehensive Biomedical Research Centre, the Newlife Foundation for disabled children, the Mothercare Group Foundation, and Mead Johnson Nutrition.

1 **Abstract**

2 **Aim:** Benefits of human breast milk (HM) in avoiding rapid infancy weight-gain and
3 later obesity could relate to its nutrient content. We tested the hypothesis that
4 differential HM total calorie content (TCC) or macronutrient contents may be
5 associated with infancy growth. **Methods:** HM hindmilk samples were collected at
6 ages 4-8 weeks from 614 mothers participating in a representative birth cohort, with
7 repeated infancy anthropometry. HM triglyceride (fat), lipid analytes and lactose
8 (carbohydrate) were measured by ¹H-NMR, and protein content by the Dumas
9 method. TCC and %macronutrients were determined. **Results:** In 614 HM samples,
10 fat content was: [median(IQR)]:2.6 (1.7-3.6)g/100mls, carbohydrate:8.6 (8.2-
11 8.8)g/100mls, protein:1.2 (1.1-1.2)g/100mls; TCC:61.8 (53.7-71.3)kcal/100mls. HM
12 of mothers exclusively breast-feeding vs. mixed-feeding was more calorific with
13 higher %fat, lower %carbohydrate and lower %protein. Higher HM TCC was
14 associated with lower 12m body mass index (BMI)/adiposity, and lower 3-12m gains
15 in weight/BMI. HM %fat was inversely related to 3-12m gains in weight, BMI and
16 adiposity, whereas %carbohydrate was positively related to these measures. HM
17 %protein was positively related to 12m BMI. **Conclusion:** HM analysis showed wide
18 variation in %macronutrients. Although data on milk intakes were unavailable, our
19 findings suggest functional relevance of HM milk composition to infant growth.

20

21 Keywords: breast milk, macronutrients, nutrition, weight, growth

22

23

24 Keynotes

- 25 • Breast feeding is associated with lower rates of infancy weight-gain and later
26 obesity, however data on breast milk composition and relationships with
27 growth are sparse.
- 28 • Macronutrient contents of 614 hindmilk samples were: fat: [median(IQR)]:2.6
29 (1.7-3.6)g/100mls, carbohydrate:8.6 (8.2-8.8)g/100mls, protein:1.2 (1.1-
30 1.2)g/100mls.
- 31 • Whilst %carbohydrate was positively related to later infant weight and
32 adiposity, %fat was inversely related, with % protein only positively related to
33 body mass index, suggesting functional implications of breast milk
34 macronutrient contents.
- 35
- 36

37 **Introduction**

38 It is increasingly recognised that early postnatal nutrition, as well as being
39 critical for optimal infancy growth, may also be associated with long-term health
40 outcomes (1). Rapid early infant weight gain predisposes to an adverse metabolic
41 phenotype in later life, with increased risk of overweight (2), central adiposity and
42 insulin resistance (3). The type of infant milk feeding (exclusive breast-feeding,
43 formula-feeding or mixing feeding), as well as specific dietary compositions and
44 volume of intake, may be important factors.

45 In contemporary western settings breast-feeding has been associated with
46 slower gains in infancy weight (4) and body fat (5), and although debated has also
47 been linked to lower risk for obesity and associated metabolic disease risk across
48 the life-course (6). It is unclear whether the slower infancy weight gain in breast-fed
49 babies is a result of lower total calorie intake or related to the nutrient composition of
50 human breast milk (HM). Previous studies documenting HM energy or macronutrient
51 contents have been recently reviewed (7) (8). Most were performed in small sample
52 sizes and few attempted to assess the influence of HM composition on subsequent
53 infancy growth outcomes.

54 The evidence on the potential effects of milk nutrient composition on infancy
55 growth is therefore almost entirely limited to trials comparing artificial infant milk
56 formulas. They report that higher milk protein concentrations increase infancy
57 weight gain and predisposition to obesity (9), but there is inconsistent evidence on
58 milk calorie contents (10, 11). In the absence of large population studies, we aimed
59 to investigate the relationships between HM total calorie content, macronutrient
60 contents, or individual lipid species and infancy growth, in a large UK birth cohort
61 study. We hypothesised that specific HM composition may be associated with
62 different patterns of weight and adiposity gain during infancy.

63

64

65

66 Patients & Methods

67 *Study Design:* The Cambridge Baby Growth Study (CBGS) is a prospective birth
68 cohort, focussing on antenatal and early postnatal determinants of infancy growth,
69 as previously described (5). Mothers were recruited during early pregnancy from a
70 single antenatal centre in Cambridge (2001-2009). The whole cohort included 1585
71 singleton, late preterm / term (gestation ≥ 36 weeks) infants with measurements at
72 birth, of whom 924 mothers were breast-feeding their infants at 8 weeks of age. A
73 subcohort of 614 mother-infant dyads, where a breast milk sample was available, is
74 described in the current report. The study was approved by the Cambridge local
75 research ethics committee, and all mothers gave informed written consent.

76

77 *Anthropometry:* Infants were measured by trained paediatric research nurses, with
78 weight, length, and skinfold thickness assessed in the newborn, and then at 3 and
79 12 months of age. Weight was measured to the nearest 1g using a Seca 757
80 electronic baby scale. Supine length was measured to the nearest 0.1cm using an
81 Infantometer (SECA 416). BMI was then calculated. Skinfold thickness was
82 measured in triplicate at four sites (triceps, subscapular, flank, quadriceps) on the
83 left hand side of the body using a Holtain Tanner/Whitehouse Skinfold Caliper
84 (Holtain Ltd).

85

86 *Breast milk collection:* To allow for comparable samples and informative
87 macronutrient analysis, mothers who breast-fed their infants were asked to hand
88 express hindmilk samples, after feeding their infant, between 4-8 weeks postnatally,
89 expressing from the same breast that they last used to feed their infant. They
90 repeated this process multiple times, keeping milk samples frozen, and a total of
91 100 mls of hindmilk was collected over a two week period, in order to reduce within-
92 day and day-to-day variations. Samples were then kept frozen at -20°C , until

93 processed at a single time point. The pooled sample was thoroughly mixed before
94 analysis.

95 Overall, infant feeding practise (exclusive breast- vs. mixed-feeding) was assessed
96 by questionnaire at age 3 months, with detailed questions about current feeding,
97 and age at starting supplementary formula milk feeds, as well as completely
98 stopping breast-feeding. From this information, infants were categorised as either
99 exclusive breast- or mixed-feeding at 8 weeks of age, contemporaneous with the
100 breast milk collections.

101

102 *Breast milk assays:* Triglyceride (fat) and lactose (carbohydrate) concentrations
103 were measured in homogenised HM samples using ¹H-Nuclear magnetic resonance
104 (NMR) spectra. To determine the lipid concentrations (in mM), 400 microlitres of a
105 homogenised HM sample was mixed with 400 microlitres CDC1₃ solvent for 10
106 minutes, and then centrifuged for 30 minutes at 10,000rpm. The non-polar fraction
107 was then used to measure lipid concentrations, from ¹H-NMR spectra. Triglyceride
108 concentration was used as a surrogate for total fat content, since this contributes
109 95-98% of total HM lipid content (12). A further ten lipid species were also
110 quantified: linoleic acid, diglycerides, monoglycerides, docosaheanoic acid,
111 18:1/16:1, esterified cholesterol, free cholesterol, total cholesterol, omega 3,
112 monounsaturated fatty acid and polyunsaturated fatty acids, as described previously
113 (13). Lactose, the major HM carbohydrate, was measured from the polar fraction of
114 the milk sample, using ¹H 1D NOESY spectroscopy. Reproducibility of the NMR
115 methods were assessed: the coefficient of variation (CV) for NMR itself was 0.03-
116 0.3% for lipid analysis, and 0.1-0.6% for analysis of polar metabolites, such as
117 lactose. Analysis of different aliquots from the same sample showed CVs of 0.3-
118 5.8% for lipids and 0.4-4.7% for the polar metabolites. NMR spectral peaks were
119 calibrated using Topspin and analysed based on previous work (14). For protein,

120 total nitrogen was measured by the Dumas method, and the protein factor
121 conversion of 6.25 used to calculate crude protein content.

122 Previous work has shown that storage conditions can potentially affect
123 macronutrient content, especially fat content due to the continued activity of lipases
124 and coalescence of fat globules (15). However, we were careful to homogenise the
125 HM samples before analysis. There was no effect of storage time on macronutrient
126 calories, %fat or %carbohydrate, however %protein was modestly positively
127 associated with the storage time [% per year, B (correlation coefficient) 0.01,
128 $p=0.01$]. For this reason analyses were adjusted for storage time using multiple
129 regression.

130

131 *Calculations:* Age and sex-appropriate standard deviation scores (SDS) were
132 calculated for infant weight, length and BMI measurements, adjusting for gestational
133 age in the newborn, by comparison to the UK 1990 growth reference (16), using the
134 LMS Pro software (17). For each of the four skinfold thicknesses an internal SDS
135 was calculated, adjusted for age, and the mean of the four skinfolds SDS was used
136 as estimate measure of adiposity in analyses.

137 The metabolisable energy content of HM was calculated using Atwater
138 conversions, taking energy contents of 4, 4 and 9 kcal/g for protein, lactose and fat,
139 respectively (18), and HM total calorie content (TCC) was then calculated as
140 kcal/100mls. The nutrient density method was used to present macronutrient
141 contents as percentages of total calorie content (i.e. %fat, %carbohydrate, and
142 %protein) (19). In order to distinguish independent effects of the individual lipid
143 species investigated, since the lipid species were highly inter-correlated (all
144 Spearman's coefficients > 0.51 , $p<0.0005$), we used a residual nutrient method:
145 each lipid concentration was regressed against the triglyceride concentration,
146 standardised residuals for each lipid species calculated and these values used in
147 subsequent analysis.

148

149 *Statistics:* The demographics of the cohort subgroup with HM samples were
150 compared to that of the entire CBGS cohort, and in particular to all mother-infant
151 pairs who were breast-feeding (either exclusively or mixed-feeding) at 8 weeks,
152 using t-tests, chi-squared tests or independent sample median tests.

153 Relationships between HM TCC, or %macronutrient contents, and infancy
154 growth were investigated, using multivariate regression models, including the
155 following variables: birthweight, gestational age, infant sex, nutrition type and HM
156 storage time. Analyses were performed using SPSS version 20, and statistical
157 significance indicated by p value <0.05.

158

159 **Results**

160 *Cohort description:*

161 The sub cohort of 614 mothers of singleton, term or later preterm infants who
162 provided a HM sample was similar to all mother-infant pairs in CBGS who were
163 breast-feeding (exclusively or in combination with formula-feeding, N=924). There
164 were no differences with respect to gestational age, maternal age, maternal pre-
165 pregnancy BMI, maternal primiparity, ethnicity, infant size at birth and subsequent
166 growth to 12 months of age. Further details of the sub cohort are shown in Table 1.

167

168 *Human milk macronutrient contents*

169 For the 614 HM samples analysed, TCC was [median (IQR)]: 61.8 (53.7-71.3)
170 kcal/100mls. The macronutrient composition was: fat (triglycerides) 2.6 (1.7-3.6)
171 g/100mls; protein 1.2 (1.1-1.2) g/100mls; carbohydrate (lactose) 8.6 (8.2-8.8)
172 g/100mls. Macronutrient contents expressed as calories per 100 mls and
173 percentages of TCC are shown in Table 2.

174 HM total calorie and macronutrient contents were unrelated to mother's pre-
175 pregnancy BMI, pregnancy weight gain, parity, gestational age at delivery or

176 socioeconomic status (assessed using home postcode-based index of multiple
177 deprivation scores as reported previously (20)), and were also unrelated to infant
178 sex (data not shown).

179

180 77% of the mothers who provided a HM sample were exclusively breast-feeding at 8
181 weeks; the others gave their infants both breast milk and infant formula milk (mixed
182 feeding). HM of exclusively breast-feeding mothers contained higher TCC [medians]
183 (62.6 vs. 58.7 kcal/100mls), higher %fat (37.6 vs. 35.0%), but lower %protein (7.3
184 vs. 8.3%) and %carbohydrate (54.7 vs. 57.5%), all $p < 0.05$. All further analyses
185 were adjusted for exclusive breast-feeding versus mixed feeding, using multivariate
186 regression modeling.

187

188 *Associations with infancy growth:*

189 As shown in Table 2, HM TCC at 4-8 weeks was inversely associated with BMI
190 ($p=0.02$) and adiposity ($p=0.008$) at age 12 months, and with 3-12 month gains in
191 weight ($p=0.02$) and BMI ($p=0.01$). With regard to %macronutrient contents (Table
192 2), HM %fat was inversely associated with BMI and adiposity at 12 months, and
193 inversely associated with 3-12 months gains in weight, BMI and adiposity. In
194 contrast, HM %carbohydrate was positively related to weight, BMI and adiposity
195 gains between 3-12 months. Figure 1 shows that the relationships between quintiles
196 of HM %fat or %carbohydrate and adiposity/BMI at 12 months were broadly linear.
197 HM %protein was positively correlated to BMI at 12 months ($p=0.04$), with no
198 association with 12 month weight or adiposity, or 3-12 month gains. Figure 1 also
199 shows adiposity/BMI for 271 exclusively formula-fed CBGS infants at the time of HM
200 sample collection, for comparison. HM %macronutrient contents showed no
201 relationships with infant length at any age.

202

203 Sensitivity analyses were carried out, separately by feeding group (exclusive breast-
204 feeding versus mixed feeding at 8 weeks). These showed the same directions of
205 associations as in the total population (full data not shown). For example, in the
206 exclusively breast-feeding subgroup (N=389), the associations with 12 month
207 adiposity were: % HM protein: B 0.02, p=0.3, % carbohydrate: B 0.009, p=0.01, %
208 fat: B -0.007, p=0.02, total calories: B -0.005, p=0.07.

209

210

211 *Human milk specific lipid species*

212

213 Concentrations of ten specific HM lipid species are shown in Table 3. In separate
214 models for each lipid species (adjusted for birthweight, gestational age, sex and
215 exclusive breast- vs. mixed-feeding), all ten lipid species showed inverse
216 associations with infant adiposity at 12 months (data not shown). Using the residual
217 nutrient method, linoleic acid was the only lipid species that remained inversely
218 related to infant adiposity at 12 months (p=0.05).

219

220 **Discussion**

221 To our knowledge, this study of 614 mother-infant pairs is the largest report
222 describing HM macronutrient contents, and the first extensive study to investigate
223 their relationships with infancy growth. We showed inverse associations between
224 HM total calorie content and subsequent gains in weight and BMI, and also later
225 adiposity. Regarding individual HM macronutrients, %carbohydrate was positively
226 correlated to subsequent infant weight, BMI and adiposity gains, whereas %fat was
227 negatively associated with these infancy outcomes. HM %protein was weakly
228 positively associated with BMI at 12 months but not gains in adiposity.

229 Associations between HM contents and infancy growth have not been
230 previously reported, largely due to the lack of other large studies. However, in

231 support of our study design, the observed positive association between HM
232 %protein content and 12m BMI is consistent with experimental evidence from large
233 clinical trials that tested isocaloric infant milk formulas containing high versus usual
234 protein contents. Unfortunately, HM intakes were not assessed in our study and
235 therefore we cannot assess whether the associations observed with HM contents
236 were mediated by nutrient intakes.

237 Of relevance, a recent study reported an inverse association between fat
238 intake at 2 years of age and body fat, assessed by bioelectrical impedance analysis
239 at 20 years (21), also suggesting that early diet containing greater fat may benefit
240 later body composition, either directly or indirectly. A higher proportion of ingested
241 carbohydrate may promote storage of glycogen and fat. Alternatively, it is possible
242 that infants fed HM with lower %fat may feel less satiated and drink larger volumes
243 of milk, hence gaining more weight. This hypothesis is supported by previous
244 observations that HM %fat was inversely related to the volume of HM intake,
245 whereas %lactose was positively correlated (22), and by older studies reporting that
246 infants consuming formula milk with lower energy, had higher dietary intakes (11).

247 A recent systematic review concluded that higher protein intake in infancy
248 and early childhood is associated with faster weight gain and greater BMI in
249 childhood (23). We did not have further detailed body composition data, making it
250 difficult to distinguish between gains in lean mass or fat mass. We found relatively
251 less inter-person variability in %protein than in other macronutrients and it may be
252 that larger differences in %protein, such as those seen in formula milk studies (9),
253 are needed to observe significant influences of protein content on infancy weight
254 gain.

255 It is difficult to directly compare our results with other previous studies of HM
256 constituents, due to differences in the timing of HM collection with pooling of
257 samples, sampling of solely hindmilk, HM assays, and the nature of the populations
258 sampled. A recent systematic review summarised the results of 'mature' HM

259 samples (taken 2-4 weeks postnatally) (7), a total of: 415 for protein, 476 for
260 carbohydrate and 567 for lipids, pooling data from a minimum of 18 studies
261 worldwide, with the largest sample size of 71 in any single study. Our study is
262 therefore far larger than any other reported, allowing informative associations with
263 HM macronutrient contents in the range of the previously pooled meta-analysed
264 values.

265 Heinig et al (1993) showed that total energy and protein intakes were
266 positively correlated to weight, not only in formula-fed infants (N=46) but also in
267 those exclusively breast-fed (N=73) (24). Specifically, total protein intake was
268 positively correlated with 3-6 month and 6-9 month weight gain in breast-fed infants.
269 Butte et al (2000) reported that intakes of HM protein, fat and carbohydrate, were all
270 positively correlated with weight gain and fat free mass gain (assessed using a
271 multicomponent body composition model) at 3-6 months, but not with fat mass gain,
272 in 40 breast-fed infants and 36 formula-fed infants (25). These studies assessed
273 intakes, not HM content, and were also much smaller cohorts, assessing
274 anthropometry at different time points, with different methods for HM collection and
275 nutrient analysis. Further, larger studies, across different populations, with
276 information on both composition and intakes are needed, using a standardised
277 sampling protocol.

278 It is interesting to note that the associations between HM macronutrient
279 contents and infant anthropometry in our study were mainly with weight, BMI and
280 adiposity, with no apparent influence on length gains. This is surprising as weight
281 gain and statural growth are closely linked in infancy, hence it may be speculated
282 that other confounders could explain the findings with adiposity. Maternal
283 characteristics could be one source of confounding. Some small previous reports
284 have shown correlations between specific maternal factors and HM fat content
285 including parity (26) and maternal anthropometric status (27). However, these
286 associations have not been extended to all macronutrients or been well replicated,

287 and we found no associations between such maternal factors and HM nutrient
288 contents. We did not assess maternal diet but other studies have reported no
289 relationship with HM contents (27).

290 Alternatively, it may be that other constituents in breast milk, such as
291 individual lipid moieties, could explain the relationships seen with HM
292 macronutrients and in particular the inverse relationships between lipid and infancy
293 adiposity. Disentangling the potential independent contribution to growth from
294 individual fatty acids, which are highly correlated with total lipid proved to be difficult.
295 Only the omega-6 fatty acid, linoleic acid showed a consistent independent inverse
296 relationship with later infancy adiposity. Of note, in the literature n-3 and n-6 long-
297 chain polyunsaturated fatty acids have received interest with respect to growth and
298 development, with for example with suggested beneficial effects on growth with
299 alpha-linoleic/DHA supplementation in developing countries, (28), however
300 generally there is an overall paucity of data for n-3 or n-6 LC-PUFAs (15-17).
301 Further detailed LC-PUFA analyses and subsequent studies are required to confirm
302 our finding, and investigate this area further.

303 The higher total calorie content found in HM from mothers who were
304 exclusively breast-feeding, when compared to those mixed-feeding, is consistent
305 with other observations, maintaining sufficient continued nutrition (29), and
306 suggesting that HM energy content may be down-regulated by infants mixed-
307 feeding. The higher %fat, with lower %protein and %carbohydrate, seen in milk of
308 mothers exclusively breast-feeding, may support our findings of growth associations
309 in indicating that this is a beneficial HM composition with regard to subsequent
310 infant adiposity. It could also be speculated that a higher %fat results in greater
311 infant satiation, resulting in continued breast-feeding, whereas hungrier babies
312 consuming HM with lower fat content are more likely to be given supplementary
313 formula milk.

314 Alternatively, there may be differences in HM production, regulated by the
315 suckling infant, or even potential confounding by the collection techniques used by
316 mothers expressing milk. Hindmilk contains more fat than foremilk (30) and
317 therefore it is not implausible that the exclusively fed infants consumed more milk,
318 and their HM samples contained relatively more hindmilk. We adjusted for exclusive
319 breast- versus mixed-feeding in our subsequent analytical models, with no
320 interaction seen between feeding type and macronutrient content in analyses, and
321 thus this issue is unlikely to confound the associations with infant growth. Similar
322 trends between HM macronutrient contents and infancy body size/growth were also
323 apparent in the exclusively breast-fed subgroup: although generally less significant
324 in this smaller group, correlations were in the same direction, and with similar effect
325 sizes.

326 Limitations of our study include the lack of information on HM intakes, and
327 therefore it was not possible to calculate total energy and macronutrient intakes. HM
328 lipid and protein contents are known to vary between individual feeds and with
329 different stages of lactation(7, 8). Mothers were encouraged to pool, over a period of
330 2 weeks, their collections of expressed hindmilk; however it is possible that
331 systematic differences existed between collections and information on the timings of
332 milk collection was not recorded. Some of these limitations will be tackled and
333 subject of further studies.

334

335 *Conclusion:* In conclusion, in this large study of HM macronutrient content, we found
336 that HM nutrient composition in early infancy differs between exclusively breast-
337 feeding and mixed-feeding mothers. Of note, HM %fat and %carbohydrate,
338 predicted changes in infancy weight and adiposity gains up to age 12 months, with
339 %protein positively related to 12 month BMI. There were no associations with length
340 gains. Although data on milk intakes were unavailable, our findings suggest that

341 higher HM %fat but lower %carbohydrate may be associated with lower gains in
342 adiposity and BMI.

343

344 **Acknowledgements:** We acknowledge the CBGS research nurses Suzanne Smith,
345 Ann-Marie Wardell & Karen Forbes. We thank all the families who contributed to the
346 study, the staff at the Addenbrooke's Wellcome Trust Clinical Research Facility, the
347 NIHR Cambridge Comprehensive Biomedical Research Centre, and the midwives at
348 the Rosie Maternity Hospital, Cambridge, UK.

349

350 **Conflict of Interest Statement:** This study received unconditional funding support
351 from Mead Johnson Nutrition. MH Schoemaker and EAF van Tol are employees of
352 Mead Johnson Nutrition. No other authors declare a conflict of interest.

353

354

355

356 **Abbreviations**

357	ALA	Alpha-linolenic acid
358	BMI	Body mass index
359	CBGS	Cambridge Baby Growth Study
360	DHA	Docosahexaenoic acid
361	LC-PUFA	Long chain polyunsaturated fatty acid
362	HM	Human breast milk
363	IQR	Interquartile range
364	m	Month
365	NMR	Nuclear magnetic resonance
366	NOESY	Nuclear Overhauser effect spectroscopy
367	SDS	Standard deviation score
368	TCC	Total calorie content

References

1. Lanigan J, Singhal A. Early nutrition and long-term health: a practical approach. *Proc Nutr Soc* 2009; 68:422-9
2. Ong KK, Loos RJ. Rapid infancy weight gain and subsequent obesity: systematic reviews and hopeful suggestions. *Acta paediatr* 2006; 95:904-8
3. Leunissen RW, Kerkhof GF, Stijnen T, Hokken-Koelega A. Timing and tempo of first-year rapid growth in relation to cardiovascular and metabolic risk profile in early adulthood. *JAMA* 2009; 301:2234-42
4. Ziegler EE. Growth of breast-fed and formula-fed infants. *Nestle Nutrition workshop series Paediatric programme* 2006; 58:51-9; discussion 9-63
5. Ong KK, Langkamp M, Ranke MB, Whitehead K, Hughes IA, Acerini CL, et al. Insulin-like growth factor I concentrations in infancy predict differential gains in body length and adiposity: the Cambridge Baby Growth Study. *Am J Clin Nutr* 2009; 90:156-61
6. Horta BL VC. Long-term effects of breastfeeding: A Systematic review. *World Health Organisation* 2013
7. Hester SN, Husted DS, Mackey AD, Singhal A, Marriage BJ. Is the macronutrient intake of formula-fed infants greater than breast-fed infants in early infancy? *J Nutr Metab* 2012;1-13
8. Reilly JJ, Ashworth S, Wells JC. Metabolisable energy consumption in the exclusively breast-fed infant aged 3-6 months from the developed world: a systematic review. *Br J Nutr* 2005; 94:56-63
9. Koletzko B, Beyer J, Brands B, Demmelmair H, Grote V, Haile G, et al. Early influences of nutrition on postnatal growth. *Nestle Nutrition Institute workshop series* 2013; 71:11-27

10. Singhal A, Kennedy K, Lanigan J, Fewtrell M, Cole TJ, Stephenson T, et al. Nutrition in infancy and long-term risk of obesity: evidence from 2 randomized controlled trials. *Am J Clin Nutr* 2010; 92:1133-44
11. Fomon SJ, Filmer LJ, Jr., Thomas LN, Anderson TA, Nelson SE. Influence of formula concentration on caloric intake and growth of normal infants. *Acta paediatr Scand* 1975; 64:172-81
12. Bittman J, Carlson SE, Couch SC, et al. Milk lipids. In: Jensen RG, editor *Handbook of Milk Composition*: Academic Press, San Diego, Calif, USA; 1995 p.495-573
13. Hoek-van den Hil EF, Keijer J, Bunschoten A, Vervoort JJ, Stankova B, Bekkenkamp M, et al. Quercetin induces hepatic lipid omega-oxidation and lowers serum lipid levels in mice. *PLoS One* 2013; 8:e51588
14. Vinaixa M, Rodriguez MA, Rull A, Beltran R, Blade C, Brezmes J, et al. Metabolomic assessment of the effect of dietary cholesterol in the progressive development of fatty liver disease. *J Proteome Res* 2010; 9:2527-38
15. Neville M. Sampling and storage of human milk. In: Jensen RG, editor *Handbook of Milk Composition*: Academic Press, San Diego, Calif, USA; 1995 p. 63-79
16. Freeman JV, Cole TJ, Chinn S, Jones PR, White EM, Preece MA. Cross sectional stature and weight reference curves for the UK, 1990. *Arch Dis Child* 1995; 73:17-24
17. Pan H CT. LMSgrowth, a Microsoft Excel add-in to access growth references based on the LMS method. Version 2.76. www.healthforallchildren.com 2011
18. de Kanashiro HC, Brown KH, Lopez de Romana G, Lopez T, Black RE. Consumption of food and nutrients by infants in Huascar (Lima), Peru. *Am J Clin Nutr* 1990; 52:995-1004
19. Willett WC, Howe GR, Kushi LH. Adjustment for total energy intake in epidemiologic studies. *Am J Clin Nutr* 1997; 65:1220S-8S; discussion 9S-31S

20. Lauzon-Guillain B, Wijndaele K, Clark M, Acerini CL, Hughes IA, Dunger DB, et al. Breastfeeding and infant temperament at age three months. *PLoS One* 2012; 7:e29326
21. Rolland-Cachera MF, Maillot M, Deheeger M, Souberbielle JC, Peneau S, Hercberg S. Association of nutrition in early life with body fat and serum leptin at adult age. *Int J Obes* 2013; 37:1116-22
22. Nommsen LA, Lovelady CA, Heinig MJ, Lonnerdal B, Dewey KG. Determinants of energy, protein, lipid, and lactose concentrations in human milk during the first 12 mo of lactation: the DARLING Study. *Am J Clin Nutr* 1991; 53:457-65
23. Hornell A, Lagstrom H, Lande B, Thorsdottir I. Protein intake from 0 to 18 years of age and its relation to health: a systematic literature review for the 5th Nordic Nutrition Recommendations. *Food & nutrition research* 2013; 57
24. Heinig MJ, Nommsen LA, Peerson JM, Lonnerdal B, Dewey KG. Energy and protein intakes of breast-fed and formula-fed infants during the first year of life and their association with growth velocity: the DARLING Study. *Am J Clin Nutr* 1993; 58:152-61
25. Butte NF, Wong WW, Hopkinson JM, Smith EO, Ellis KJ. Infant feeding mode affects early growth and body composition. *Pediatrics* 2000; 106:1355-66
26. Jensen RG. Miscellaneous factors affecting composition and volume of human and bovine milks. In: Jensen RG, editor *Handbook of Milk Composition*: Academic Press, San Diego, Calif, USA; 1995 p. 237-72
27. Villalpando S, del Prado M. Interrelation among dietary energy and fat intakes, maternal body fatness, and milk total lipid in humans. *J Mammary Gland Biol Neoplasia* 1999; 4:285-95
28. Huffman SL, Harika RK, Eilander A, Osendarp SJ. Essential fats: how do they affect growth and development of infants and young children in developing countries? A literature review. *Matern Child Nutr* 2011; 7 Suppl 3:44-65

29. Wells JC, Jonsdottir OH, Hibberd PL, Fewtrell MS, Thorsdottir I, Eaton S, et al. Randomized controlled trial of 4 compared with 6 mo of exclusive breastfeeding in Iceland: differences in breast-milk intake by stable-isotope probe. *Am J Clin Nutr* 2012; 96:73-9
30. Jensen RG. The lipids in human milk. *Prog Lipid Res*. 1996; 35:53-92

Table 1: Description of the study members who provided a breast milk sample, in relation to the wider Cambridge Baby Growth Study cohort. Median & IQR are displayed.

	Mothers providing HM samples (N=614)	All CBGS mothers exclusively breast- & mixed-feeding (N=924)
Demographics		
Gestational age (weeks)	40.1 (39.1-41.0)	40.0 (39.1-41.0)
Maternal age (years)	33.9 (31.1-36.5)	34.0 (31.2-36.5)
Maternal BMI (kg/m ²)	22.8 (20.9-25.2)	22.7 (20.8-25.2)
Index of deprivation	9.0 (6.9-9.0)	9.0 (6.8-9.0)
Maternal primiparity (%)	43%	42%
White Caucasian (%)	96%	96%
Infant sex (% male)	51%	51%
Exclusive breast-feeding (%)	73%	77%
Growth data		
Birth		
Weight (kg)	3.56 (3.22-3.87)	3.55 (3.22-3.85)
Length (cm)	51.5 (50.0-53.5)	51.5 (50.0-53.3)
Mean skinfold thickness (mm)	6.2 (5.3-7.4)	6.1 (5.2-7.3)
BMI (kg/m ²)	13.3 (12.2-14.3)	13.3 (12.2-14.2)
3 months		
Weight (kg)	6.10 (5.60-6.64)	6.09 (5.59-6.62)
Length (cm)	61.2 (59.4-63.0)	61.2 (59.5-63.0)
Mean skinfold thickness (mm)	10.8 (9.4-11.9)	10.7 (9.4-11.9)
BMI (kg/m ²)	16.3 (15.4-17.2)	16.2 (15.3-17.2)
12 months		
Weight (kg)	9.85 (9.10-10.60)	9.88 (9.15-10.60)
Length (cm)	75.8 (74.0-77.7)	75.6 (73.9-77.7)
Mean skinfold thickness (mm)	11.0 (9.8-12.5)	11.0 (9.7-12.4)
BMI (kg/m ²)	17.1 (16.2-18.0)	17.1 (16.3-18.1)

Table 2: Human milk macronutrient contents and their associations with infancy growth (based on N=614 samples).

	Fat	Carbohydrate	Protein	Total calorie content
<i>Macronutrient contents*</i>				
Calories (kcal) per 100 mls	23.1 (15.4-32.4)	34.3 (32.9-35.3)	4.6 (4.2-5.1)	
%macronutrient content ¹	37.3 (28.4-48.9)	55.2 (47.6-62.9)	7.5 (6.4-9.0)	
<i>Associations with growth</i>				
Weight SDS at 3 mo	B -0.001, p=0.7	B 0.001, p=0.8	B 0.02, p=0.3	B -0.002, p=0.4
Weight SDS at 12 mo	B -0.005, p=0.1	B 0.006, p=0.2	B 0.03, p=0.1	B -0.005, p=0.1
Delta weight SDS 3-12 mo	B -0.007, p=0.02	B 0.008, p=0.02	B 0.03, p=0.1	B -0.006, p=0.02
Mean skinfolds SDS at 3 mo	B -0.004, p=0.2	B 0.004, p=0.1	B 0.02, p=0.3	B -0.003, p=0.3
Mean skinfolds SDS at 12 mo	B -0.009, p=0.001	B 0.01, p<0.0005	B 0.03, p=0.1	B -0.007, p=0.008
Delta skinfolds SDS 3-12 mo	B -0.007, p=0.04	B 0.008, p=0.03	B 0.02, p=0.4	B -0.005, p=0.08
BMI SDS at 3 mo	B -0.004, p=0.3	B 0.004, p=0.3	B 0.02, p=0.4	B -0.002, p=0.4
BMI SDS at 12 mo	B -0.01, p=0.002	B 0.01, p=0.002	B 0.04, p=0.04	B -0.008, p=0.02
Delta BMI SDS 3-12 mo	B -0.01, p=0.005	B 0.01, p=0.005	B 0.04, p=0.08	B -0.008, p=0.01
Length SDS at 3 mo	B 0.002, p=0.5	B -0.003, p=0.4	B 0.01, p=0.5	B -0.001, p=0.6
Length SDS at 12 mo	B 0.004, p=0.3	B -0.005, p=0.2	B 0.003, p=0.9	B 0.000, p=0.9
Delta length SDS 3-12 mo	B 0.001, p=0.6	B -0.003, p=0.6	B -0.005, p=0.7	B 0.001, p=0.6

*median (IQR)

¹ %macronutrient was calculated as macronutrient energy / total energy content

Models were adjusted for exclusive breast- vs. mixed feeding at 8 weeks, sex, GA, birthweight, duration of sample storage

Table 3: Concentrations of human milk lipid species (mmol/ L) N=614

Lipid species	Median (IQR) mmol/ L
Linoleic acid	6.62 (4.39-9.17)
Diglycerides	1.95 (1.19-3.02)
Monoglycerides	0.63 (0.38-0.94)
DHA	0.32 (0.22-0.44)
18:1/16:1	0.77 (0.53-1.08)
Esterified cholesterol	0.18 (0.13-0.24)
Free cholesterol	0.20 (0.14-0.29)
Total cholesterol	0.37 (0.26-0.49)
Omega 3	2.48 (1.62-3.53)
MUFA & PUFA	80.96 (54.66-110.56)

DHA: Docosahexaenoic acid

18:1/16:1 : Oleic/palmitoleic acid

MUFA & PUFA: Monounsaturated fatty acid & polyunsaturated fatty acid

Figure 1: Infant adiposity at 12 months by quintiles of human milk macronutrient contents at 4-8 weeks.

a) 12 month SF SDS: skinfold standard deviation score as the mean SDS of measurements at four sites. Error bars indicate group means & 95% confidence intervals.

b) 12 month BMI SDS. Error bars indicate group means & 95% confidence intervals.