Effects of feeding term infants low energy low protein formula supplemented with bovine milk fat globule membranes.

Niklas Timby
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Abstract

Background Observational studies have shown that early nutrition influences short- and long-term health of infants. Formula-fed infants have higher protein and energy intakes and lower intakes of several biologically active components present in human milk. Some of these are present in the milk fat globule membrane (MFGM). The aim of the present study was to examine the effects of feeding term infants an experimental low energy low protein formula supplemented with bovine milk fat globule membranes. Our hypothesis was that infants fed experimental formula (EF), compared to infants fed standard formula (SF), would have outcomes more similar to a breast-fed reference (BFR) group.

Methods In a double-blinded randomized controlled trial, 160 exclusively formula-fed, healthy, term infants were randomized to receive EF or SF from <2 to 6 months of age. A BFR group consisted of 80 breast-fed infants. Measurements were made at baseline, 4, 6 and 12 months of age. The EF had lower energy (60 vs. 66 kcal/100 mL) and protein (1.20 vs. 1.27 g/100 mL) concentrations, and was supplemented with a bovine MFGM concentrate.

Results At 12 months of age, the EF group performed better than the SF group in the cognitive domain of Bayley Scales of Infant Development, 3rd Ed. During the intervention, the EF group had a lower incidence of acute otitis media than the SF group, less use of antipyretics and the EF and SF groups differed in concentrations of s-IgG against pneumococci. The formula-fed infants regulated their intakes by increasing meal volumes. Thus, there were no differences between the EF and SF groups in energy or protein intakes, blood urea nitrogen, insulin or growth including body fat percent until 12 months of age. Pressure-to-eat score at 12 months of age was reported lower by parents of formula-fed infants than by parents of breast-fed infants, indicating a low level of parental control of feeding in the formula-fed groups. Neither high pressure-to-eat score nor high restrictive score was associated with formula feeding. During the intervention, the EF group gradually reached higher serum cholesterol concentrations than the SF group, and closer to the BFR group. At 4 months of age, there was no significant difference in the prevalence of lactobacilli in saliva between the EF and SF groups.

Conclusions Supplementation of infant formula with a bovine MFGM fraction enhanced both cognitive and immunological development in formula-fed infants. Further, the intervention narrowed the gap in serum cholesterol concentrations between formula-fed and breast-fed infants. The lower energy and protein concentrations of the EF were totally compensated for by a high level of self-regulation of intake which might, at least partly, be explained by a low level of parental control of feeding in the study population. The findings are of importance for further development of infant formulas and may contribute to improved short- and long-term health outcomes for formula-fed infants.
Original papers


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<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
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<tbody>
<tr>
<td>AOM</td>
<td>acute otitis media</td>
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<tr>
<td>ARA</td>
<td>arachidonic acid</td>
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<tr>
<td>Bayley-III</td>
<td>Bayley Scales of Infant and Toddler Development, Third Edition</td>
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<td>BFR</td>
<td>breast-fed reference</td>
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<td>BMI</td>
<td>body mass index</td>
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<td>BUN</td>
<td>blood urea nitrogen</td>
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<td>DHA</td>
<td>docosahexaenoic acid</td>
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<td>EEG</td>
<td>electroencephalogram</td>
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<tr>
<td>EF</td>
<td>experimental formula</td>
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<td>ESPGHAN</td>
<td>The European Society for Paediatric Gastroenterology, Hepatology and Nutrition</td>
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<tr>
<td>hsCRP</td>
<td>high sensitive C-reactive protein</td>
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<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
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<tr>
<td>LCPUFA</td>
<td>long chain polyunsaturated fatty acids</td>
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<td>MFGM</td>
<td>milk fat globule membranes</td>
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<td>SF</td>
<td>standard formula</td>
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Sammanfattning på svenska

Bakgrund

Från födseln fram till att man börjar med smakportioner, vanligen mellan 4-6 månadars ålder, får barn som helamas all sin näring via bröstmjölken vars sammansättning regleras i moderns bröstkörtel. Barn som inte ammas bör istället få modersmjölsersättning som då blir den enda näringskällan. Modersmjölsersättning baseras oftast på komjöl och eftersom varje art har sin specifika sammansättning av mjölk, behöver komjölken ändras i sin sammansättning för att ge en tillfredsställande näringsstillsförsel. Ersättningen ska svara mot näringsbehovet för varje spädbarn som får den och design av modersmjölsersättning, utan hjälp av regleringen i bröstkörteln, är en av de största utmaningarna inom livsmedelsproduktion. Faktorer som saknas eller finns i fel koncentration kan innebära negativa hälsoeffekter.

Förbättringar av modersmjölsersättningar ska baseras på uppmätta skillnader mellan ammade och ersättningsuppfödda barn. De skillnader man ser är små och på individnivå oftast betydelselösa men på gruppnivå finns klart mätbara skillnader som kan ha hälsoeffekter i befolkningen, såsom skillnader i tillväxt, kognitiv utveckling och sjuklighet i infektioner. Amning tycks också ha en skyddande effekt mot diabetes typ 1 och celiaki. Under de senaste decennierna har konceptet tidig metabol programmering fått uppmärksamhet där man kan koppla tidiga miljöfaktorer påverkan på metabolasystem i kroppen till långsiktiga hälsoeffekter. Tidig nutrition och tillväxt är några faktorer som har föreslagits kunna ha programmeringeffekter som kan påverka risk för fetma, det metabolas syndromet och hjärt-kärlsjukdomar i vuxen ålder.

Eftersom det är oetiskt att randomisera barn till amning eller modersmjölsersättning är alla studier som jämför skillnaderna i observatiedstudier. Det innebär att det alltid finns en möjlighet att de skillnader man ser beror på skillnader mellan grupperna som inte har med uppfödningssätt att göra. Mycket talar dock för att åtminstone en del av skillnaderna orsakas av skillnader i sammansättning mellan modersmjölsersättning och bröstmjölk, där bröstmjölk har lägre energi- och proteininhåll och innehåller bioaktiva ämnens som saknas i modersmjölsersättning. En bioaktiv fraktion i bröstmjölk är mjölkfettsmembranet (MFGM) som innehåller bl a glykoproteiner och fosfolipider som både har immunologiska effekter och är byggstenar i nervsystemet. MFGM saknas i modersmjölsersättning eftersom vegetabiliskt fett, och inte mjölkfett, används som fettkälla. Nyligen har renade MFGM-fraktioner från komjöl börjat produceras kommersiellt.

Hypotes

Vår huvudhypotes var tvådelad: 1) att en experimentell modersmjölsersättning (EF) med sänkt energi- och proteininnehåll skulle ge ett lägre energi- och proteinintag och däremellan barn som fått EF skulle ha ett tillväxtnärmare närmare en ammad referensgrupp (BFR) jämfört med barn som fått standard-modersmjölsersättning (SF) och 2) att tillsats av en MFGM-fraktion från komjölk till EF skulle påverka neurologisk utveckling positivt för barn som fått EF. För de sekundära utfallsvariablerna var vår hypotes att barn som fått EF, jämfört med barn som fått SF, skulle få utfall närmare BFR-gruppen i riskmarkörer för hjärt-kärlsjukdom, infektionssjukheter och kolonisation av munfloran med laktabacillar.

Metoder

I en dubbelblindad studie randomiserades 160 barn som slutat ammas innan 2 månaders ålder till att få EF eller SF fram till 6 månaders ålder. En ammad referensgrupp (BFR) med 80 barn rekryterades parallellt. Besök gjordes vid inklusionen (<2 mån), 4, 6 och 12 månaders ålder. EF innehöll mindre energi (60 kcal/100 mL) och protein (1.2 g/100 mL) än SF samt hade tillsats av en MFGM-fraktion från komjölk.

Resultat

Vid 12 månaders ålder hade EF-gruppen i medeltal högre resultat på den kognitiva domänen av testinstrumentet Bayley III.

Under interventionen hade EF-gruppen signifikant lägre incidens av akut öroninflammation än SF-gruppen, mindre användande av febermedicin och skilde sig i nivåer av antikroppar mot pneumokocker.

Föräldrarna till de ersättningsuppfödda barnen rapporterade lägre “pressure-to-eat score”, ett mått på föräldrarnas oro för undervikt och vilja att påverka barnets intag, vid 12 månader jämfört med föräldrarna till BFR-gruppen tydande på en låg grad av föräldrakontroll för de ersättningsuppfödda grupperna.

Under interventionen nådde EF-gruppen gradvis högre serum-kolesterolnivåer än SF-gruppen och det var ingen signifikant skillnad mellan EF- och BFR-grupperna vid 6 månaders ålder.

Vid 4 månaders ålder hade BFR-gruppen oftare laktobaciller i sin munflora jämfört med EF- och SF-grupperna.

**Slutsatser**

Tillsats med MFGM till modersmjölsersättning gav en positiv effekt både på den kognitiva och immunologiska utvecklingen hos ersättningsuppfödda barn. Interventionen minskade också skillnader i kolesterolkonzentrationer mellan ersättningsuppfödda och ammade barn.

Det sänkta energi- och proteininnehållet i EF kompenserades fullständigt av de ersättningsuppfödda barnens egenreglering. Den oväntat goda egenregleringen kan åtminstone delvis förklaras av låg grad av föräldrakontroll i vår studiepopulation.

Resultaten är en viktig del i det fortsatta arbetet med utveckling av modersmjölsersättningar och skulle kunna bidra till att förbättra ersättningsuppfödda barns hälsa på kort och lång sikt.
Background

The nutritional support needed for growth and development of the fetus and infant is based on the mother’s diet and supplied via the placenta and breast milk. When introduced to complementary food, typically between 4 and 6 months of age, the infant’s diet becomes more and more diversified and is expected to meet the needs for growth and development independently of the mother’s diet. When breast milk is not available for the infant, the nutritional support during the nursing period is solely dependent on the composition and amount of the infant formula given and, therefore, the functional composition of an infant formula is one of the greatest challenges in nutrition. The composition of the formula has to meet the need for every infant fed the formula, without help from the regulation in the mother’s mammary gland. Any compound with too low or too high concentration may result in unfavourable functional outcomes for the recipient infant.

Figure 1. Breast-feeding vs. formula-feeding.
How to improve infant formulas?

The European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) Committee on Nutrition has commented on how future work on improvement of infant formulas should be carried out. The Committee stresses that the composition of human milk should not be the reference in studies on infant formulas, but the outcome of healthy breast-fed infants. A systematic review of existing information should be the base for the introduction of any modifications to infant formulas, and appropriate studies of efficacy and safety should be performed. Blind randomization between study and control formula is important (1).

Observed differences between formula-fed and breast-fed infants

As randomization between formula-feeding and breast-feeding is unethical, the knowledge on differences between formula-fed and breast-fed infants is based on observational studies. In all populations, there are sociodemographic differences between the parents of formula-fed and breast-fed infants and different studies adjust for these differences in different ways. Due to the observational design of the studies, it is uncertain if any found difference is caused by differences in the composition of infant formula and breast milk or due to differences in background factors not adequately adjusted for.

Early growth and protein intake

The protein content of infant formula has always been higher than that of breast milk, mainly to ensure sufficient levels of essential amino acids. With improved protein quality, the protein concentration has gradually been lowered. In the DARLING study from the late 1980s, formula-fed infants showed a more rapid gain in weight and lean body mass between 3 and 9 months of age, a significantly higher total energy intake and 70% higher protein intake compared to breast-fed infants (2). In a large European multi-center study recruiting infants between 2002 and 2004, protein: energy ratios in iso-caloric infant and follow-on formulas were reduced from 2.9 to 1.8 and from 4.4 to 2.2 g/100 kcal, respectively. This resulted in a reduction of weight-for-length z-score at 2 years by 0.20 in the low protein compared to the high protein group. However, the low protein group still had significantly higher weight and BMI than a breast-fed reference group at 2
years of age (3). The protein intake has been suggested to be the most important macronutrient factor for the accelerated growth in formula-fed infants since high protein intake leads to high levels of growth-promoting hormones like insulin and IGF-1 (4), and higher protein intake has been linked to future overweight (5, 6).

Regulation of energy and volume intake

The average energy density of mature breast milk has long been considered to be about 67 kcal/100 ml and virtually all infant formulas have the same energy density. Current regulations of the composition of infant formulas (7-10) are based on this assumption, albeit with some allowance for variation, resulting in a minimum of 60-67 kcal/100 ml (8, 10, 11). However, several studies strongly suggest that the metabolizable energy of breast milk is considerably lower, and possibly over-estimated by 10-30% (12-14).

It has been reported that healthy infants have the capacity to self-regulate meal size depending on energy density of the food during the first year (15), and this was noted already during the first week in premature newborns (16). However, early studies on infants fed formulas with large differences in energy density have shown that 1.5 – 3 months old infants were able to fully regulate the intake of a low and high energy formula (54 vs 100 kcal/100 ml), but the regulation was incomplete before 1.5 months of age (17). Infants 4-6 months who received a very low energy formula (36 vs 67 kcal/100 ml) plus unlimited complementary food were not able to fully up-regulate the volume intake and had a lower energy intake and weight gain than infants fed standard formula (18).

If the infant’s self-regulation of energy intake is disturbed, the growth pattern can be affected. Both parental handling of the feeding dynamic and intrinsic characteristics of the child (“appetite”) influence weight gain in early infancy (19). The temperament of the newborn infant affects weight gain; “fear” (rejection of new objects or people) is associated with poor weight gain and “distress to limitations” (negative emotionality and the infant’s reactions to frustrating situations) with fast weight gain (20). Several studies have shown a difference in self-regulation between formula-fed and breast-fed infants suggesting that formula-feeding is a risk factor for disturbed self-regulation. Data on milk intake for breast-fed infants are available from stable isotope methodology and shows an increase in volumes from 1 to 4 months reaching a plateau after 6 months with a great variability within and between populations (21). Bottle-fed infants showed less
variability in volume between feedings, an earlier daytime-concentration of feedings and larger total daily volume of feeding (22). Further, children who were bottle-fed in early infancy had reduced self-regulation in late infancy (23). Mothers who had breast-fed their infants showed less restrictive behavior regarding child feeding at 1 year compared to mothers who had exclusively formula-fed their infants (24). A high level of maternal control at 6 months of age worsens both underweight (“failure to thrive”) and overweight problems between 6 and 12 months of age (25), and the level of maternal control explains 13% of the association between breast-feeding duration and BMI at 3 years of age (26). In rats, maternal behavior has been shown to result in epigenetic changes in the pups resulting in an altered response to stress (27). Intake of formula can also be affected by the concentration of certain nutrients, e.g. supplementation of formula with free glutamate decreases volume intake (28).

**Neurodevelopment**

Experimental data from animal models and short-term follow-up studies indicate a relationship between early nutrition and neurodevelopment (29). Many nutrients affect the development of the nervous system in early childhood including protein, energy, fat, iron, zinc, copper, iodine, selenium, vitamin A, choline and folate. Deficits in certain nutrients can have either global or circuit-specific negative effects on brain development (30), and the importance of choosing the right measurements of neural function in nutrition-cognition studies has been stressed (31). Breast-fed and formula-fed infants have been shown to differ in both global and specific areas. For preterm infants, total brain volume and especially the white matter volume in late childhood is positively correlated to the amount of breast milk ingested during the first month (32). During the first year, healthy breast-fed and formula-fed infants have different developmental profiles of EEG spectral power (33). Several large studies have shown better psychomotor development and cognitive function in breast-fed compared to formula-fed infants. In a meta-analysis of observational studies adjusting for potential confounders (socioeconomic, educational and hereditary factors), a difference of 3.16 points in cognitive testing to the breast-fed children’s advantage was found at 6-23 months of age. The difference was persistent at least until 15 years of age. The advantage increased with duration of breast-feeding and low birth weight infants benefitted most (34). Due to the observational design of the included studies, the external validity of the meta-analysis is controversial, though (35, 36). However, one single randomized study from the 1980’s showed higher developmental scores in
preterm infants receiving banked breast-milk supporting the hypothesis that there are factors in breast milk that enhance neural development (37). In the PROBIT study in Belarus where the rate of breast-feeding was low, hospitals were cluster randomized to breast-feeding promotion or no intervention. The breast-feeding promotion significantly increased the rate of breast-feeding and was associated with 7.5 points higher verbal IQ at 6.5 years (38).

Several factors have been suggested to explain the difference in neurodevelopment between formula-fed and breast-fed infants. Any candidate factor should be present at a different concentration in breast milk than in formula, and have an effect on neurodevelopment shown in animal or human studies. Supplementation with the long chain polyunsaturated fatty acids (LCPUFA) docosahexaenoic acid (DHA) and arachidonic acid (ARA) to infant formula has been subjected to several randomized controlled trials. However, Cochrane reviews have not confirmed that LCPUFA supplementation has a positive effect on neurodevelopment neither in term, nor in preterm infants (39, 40), leaving the field open for other candidate factors. Children aged 4-6 years who were breast-fed as infants have a greater likelihood of foveal stereo acuity at 4-6 years compared to those who were fed formula with or without DHA supplementation, further suggesting that breast milk factors besides DHA might be of importance for visual development (41).

Sphingomyelin, choline, sialic acid, gangliosides and cholesterol are all present in higher concentration in human milk than in infant formula (42-45). Sphingomyelin-fortified milk had a positive association with neurobehavioral development in very low birth weight infants (46). Choline supplementation improved memory and learning in rats (47). Sialic acid is important for the development and growth of neural tissue (48), and supplementation with sialic acid in a pig model improved memory and learning (49). Gangliosides are known to play a role in neuronal growth, migration and maturation, neuritogenesis, synaptogenesis, and myelination (50). One double-blinded randomized study showed a positive effect of supplementation with complex lipids containing gangliosides to infant formula, from 2 to 8 weeks until 24 weeks of age, on cognitive function measured with Griffith Mental Development Scale at 24 weeks of age (51). Cholesterol increases myelination in the mouse brain (52) and is protective against neural degradation in elderly humans (53). The concentration of iron is considerably higher in infant formula than in human milk, and the results from one study suggests that too much iron to iron replete infants may have a negative effect on neurodevelopment (54).
Infections and immunology

The preventive effect of breast-feeding on infections, especially in developing countries, has been pointed out as its most important health benefit (55). Even in developed countries, formula-fed infants have higher risk of infections during the first year of life, e.g. respiratory tract infections (56), acute otitis media (AOM) (57, 58) and gastroenteritis (59), compared to breast-fed infants and promoting breast-feeding has been shown to reduce infectious diseases at the community level (60). Several antibacterial, antiviral and immune-modulating factors in human milk have been described; proteins including immunoglobulins, cytokines, lactoferrin, lysozyme, κ-casein, lactoperoxidase, haptocorrin, α-lactalbumin, osteoprotegerin and bile salt-stimulated lipase (61-65), but also oligosaccharides, glycoconjugates (66) and lipids (67, 68). These factors can act directly by intervening with the adhesion of microbes to the mucosa, by killing the pathogenic microbes, by modulating the intestinal microbiota, or by affecting other parts of the systemic immune system (69-73).

Breast-fed and formula-fed infants differ in their immune responses (74) where breast-feeding is associated with an anti-inflammatory cytokine milieu (75), Th1 type response (76) and better serological protection after vaccination (77). Infant feeding and weight gain pattern during the first year of life has been shown to influence the risk of type 1 diabetes where breastfeeding seems to be protective (78). Breastfeeding also seems to reduce the risk of developing celiac disease (79).

Cardiovascular disease

Observational studies on the relationships between perinatal factors and health or disease in adult life have given rise to the concept of metabolic programming resulting from environmental exposures during certain early time windows causing irreversible effects on the individual that remain through life. The suggested possible mechanisms are many, from injuries causing suboptimal development of organs to epigenetic changes resulting in modified expression of genes. Early feeding is one important factor affecting metabolic programming. Many observed relationships are related to metabolic factors or growth, highly dependent on early diet, even in previous generations or during pregnancy or early infancy.

Barker et al. found that low birth weight is a risk factor for ischemic heart disease later in life (80). The fetal origins hypothesis stated that fetal under-
nutrition leads to disproportionate fetal growth and programs later coronary heart disease (81). The same phenomenon was found for type 2 diabetes mellitus which led to the thrifty phenotype hypothesis (82). Besides fetal environmental factors, a genetic predisposition of insulin resistance giving both an intrauterine growth restriction and later insulin resistance has been proposed to be a part of the explanation of the association between low birth weight and later metabolic syndrome (83). Follow-up of children to mothers who were pregnant during the Dutch famine 1944-1945 has shown that the timing of the nutritional insult is important; starvation during early gestation was associated with more coronary heart disease, raised blood lipids, altered clotting and obesity while mid-gestation starvation was associated with obstructive airway disease and micro-albuminuria and late-gestation starvation with decreased glucose tolerance (84-86). Even a trans-generational effect has been reported: children of fetuses exposed to in utero starvation during the Dutch famine suffer from neonatal obesity and poor health later in life (87).

The findings of the importance of early growth velocity, where a relationship between fast growth in childhood and cardiovascular disease (88), impaired glucose tolerance (89), obesity (90), increased blood pressure (91, 92) and poor endothelial function (93), further contributed to the programming concept. Singhal and Lucas formulated the growth acceleration hypothesis, i.e. that accelerated growth in childhood is the common denominator for programming cardiovascular disease (94). The growth acceleration can start already in fetal life and high maternal weight gain during pregnancy increases birth weight independently of genetic factors (95). Another way of describing the idea of metabolic programming is an imbalance between on one hand the metabolic capacity which is set in fetal life and infancy, and on the other hand the metabolic load which is mainly dependent on the growth pattern from infancy to adulthood (96).

The mechanisms responsible for the changes in central circuits and peripheral tissues are not fully understood (97). From a molecular point of view, epigenetic changes resulting in altered expression of certain genes are likely to play a role (98). From the phenotypical point of view, changes in the development and organization of hypothalamic circuits regulating body weight and energy balance are probably important (99) besides the possible changes in peripheral tissues listed below:

- Children born small for gestational age with catch-up growth have elevated insulin levels and increased proportion of body fat, characteristics probably important for any programming effect of
accelerated early growth (100). Increased body fat is partly mediated by suppressed thermogenesis (101).

- **Inflammation** plays a key role in the development of atherosclerosis, and increased levels of several inflammatory markers are associated with cardiovascular disease, e.g. C-reactive protein (CRP), tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) (102, 103). CRP itself can also be a direct mediator of endothelial injury (104). Children and young adults aged 5-25 years of parents with essential hypertension had higher CRP-levels than offsprings to normotensive parents (105).

- **Elevated homocysteine levels** are hypothesized to cause toxic endothelial damage resulting in both reactive oxygen species inducing oxidative damage to endothelial cells and decreasing endothelial production of nitric oxide leading to impaired vascular reactivity and platelet activation and thrombosis (104). In children with familial hypercholesterolemia, total homocysteine is related to carotid intima-media thickness (106). Children born small for gestational age have elevated homocysteine levels at 8-13 years (107).

- Overweight children aged 5-18 years have an *unfavorable blood lipid profile*, as do adults with metabolic syndrome, i.e. high total cholesterol, high low-density lipoprotein cholesterol, low high-density lipoprotein cholesterol and high triglycerides (108).

- In children born small for gestational age, low serum adiponectin is associated with insulin resistance at 12 years (109), and with elevated leptin at 8-13 years (107).

- Chronic stress is linked to metabolic syndrome in a dose-dependent manner (110). Both elevated cortisol levels and autonomic dysfunction are involved (111). In infants born small for gestational age, *increased glucocorticoid activity* is associated with insulin resistance at 12 years (109).

- Overweight children with metabolic syndrome have elevated levels of 8-iso-prostanate, a marker for *systemic oxidative stress* (112).

- **Blood pressure** is significantly correlated to fasting insulin in children 3-18 years, and fasting insulin levels predict the level of blood pressure 6 years after (113). Systolic blood pressure in childhood has been shown to predict hypertension and risk of metabolic syndrome in adulthood (114).

- Children with pulmonary hypertension have elevated plasma nitrate levels (115). *Nitric oxide* (NO) is the key molecule in the relaxation of smooth muscles in the vascular wall and the exogenous pathway from dietary nitrate and nitrite and the reduction of nitrate to nitrite
by bacterial nitrate reductases in the oral cavity has been suggested as a potential factor affecting cardiovascular disease (116). *Veillonella spp.* is the most prevalent nitrate reductase positive taxa in the oral cavity (117).

- **Vascular changes** in blood vessels associated with the development of atherosclerosis can be found in early childhood. Anatomic (intima-media thickness) as well as physiologic (flow-mediated dilation and endothelial performance) and mechanical (arterial distensibility) changes can be measured with a non-invasive ultrasound technique (104). Aortic intima-media thickness was greater in newborn infants with low birth weight compared to normal birth weight babies. Aortic intima-media thickness was also greater in neonates of smoking mothers (118). Pre-pubertal children with obesity had an increased carotid intima-media thickness, which decreased after weight loss (119). Children aged 10-19 years with familial hypercholesterolemia had greater carotid intima-media thickness than a control group (106).

- Differences in the infant’s intestinal flora predict later overweight (120), and has been suggested as an important risk factor for obesity (121, 122). On a group level, there are differences in the colonization pattern of the gastrointestinal tract between breast-fed and formula-fed infants. In the oral flora, *lactobacilli* are more common in breast-fed infants (123). In the gut flora, *L. rhamnosus, clostridia, bacterioides, enterococi* and *enterobacteriaceae* are more common in formula-fed infants, while *staphylococci* are more common in breast-fed infants (124).

- A twin study has shown that both genetic and environmental factors can explain the clustering of metabolic factors in metabolic syndrome (125). Children aged 11-15 years with at least one parent with metabolic syndrome had lower glucose disposal in a euglycemic-hyperinsulinemic clamp and higher fasting insulin levels compared with children with parents without metabolic syndrome (126). Children of parents with early coronary artery disease were overweight in childhood and had unfavourable cardiovascular risk factors profiles (127). Mitochondrial DNA abnormalities are related to both placental dysfunction and vascular changes in atherosclerosis and have been considered as a possible genetic/intrauterine environmental factor explaining metabolic programming effects (128). Both maternal and fetal genotypes have been associated to early infant growth (129). Children with genetic markers of known adult obesity risk genes show faster weight and
length gain during the first 6 weeks of life, suggesting that the genetic risk profile affects growth directly after birth (130).

According to the growth acceleration hypothesis (94), formula-fed infants are at risk of future cardiovascular disease, because of a more rapid weight gain compared to breast-fed infants (131, 132). A relation between formula-feeding and higher risk for cardiovascular disease has been suggested from several studies. There is a dose-dependent association between longer duration of breast-feeding and decreased risk of overweight (133). According to Owen et al., adults that were formula-fed had 39% higher risk of type 2 diabetes (134), 13% higher risk of obesity (135) and 1.1 mm Hg higher systolic blood pressure (136). Breastfed infants had higher blood cholesterol during infancy but lower levels in adulthood (137). Another study showed that breastfed infants had higher cardiorespiratory fitness in childhood and adolescence even after correction for background factors, weight and physical activity (138). Other possible programming mechanisms have not been shown to be related to infant feeding mode, for example it should be noted that one large study could not find any association between breastfeeding in infancy and inflammatory status in adolescence (139).

**The next step to improve infant formula, the rationale for the present study**

The most obvious differences in developed countries between formula-fed and breast-fed infants found in observational studies are the differences in growth pattern, cognitive development and infections, particulary AOM. This indicates that lowering energy and protein intakes of formula-fed infants aiming at mimicking the growth of breast-fed infants and to add functional components similar to those in breast milk to infant formula to enhance neurodevelopment and the defense against infections would be the way to further narrow the gap in performance between formula-fed and breast-fed infants.

**The milk fat globule membrane**

The lactating mammary gland cell forms and releases lipids by a unique mechanism. In the cytoplasm of the epithelial cells, droplets of the hydrophobe triacylglycerol core are surrounded by a coating phospholipid/cholesterol layer with incorporated proteins. These lipid droplets are secreted from the cells by fusion with the apical plasma
membrane or by exocytosis over the apical plasma membrane after being surrounded by secretory vesicles. This gives the lipid droplet in the lumen a triple phospholipid layer membrane consisting of the coating layer and the double phospholipid/cholesterol layer with proteins and glycoproteins derived from the cell membrane or secretory vesicles. The proteins are located to different layers in the membranes with the carbohydrates of the glycoproteins directed outwards on the hydrophilic surface of the lipid droplet. The membrane surrounding the secreted fat droplets is called the milk fat globule membrane (MFGM). The lipid: protein weight ratio of the MFGM is approximately 1:1 (140, 141).

The lipid fraction of the MFGM is rich in various phospholipids and cholesterol. Phospholipids make up 30% of the total lipid weight. Sphingomyelin, phosphatidylcholine and phosphatidylethanolamine make up 30% each of the phospholipid content in MFGM (140).

Choline is a highly methylated compound and is a precursor for the biosynthesis of the membrane constituents phosphatidylcholine, sphingomyelin and choline-containing plasmalogens as well as the neurotransmitter acetylcholine. In foods, choline exists unesterified or esterified as phosphocholine, glycerophosphocholine, phosphatidylcholine and sphingomyelin. The choline and choline ester content is different in breast milk than in infant formula; the sphingomyelin and phosphocholine concentrations being higher in breast milk (142). Choline is closely related to folate and is, like folate, essential for the development of the nervous system. The fetus and the neonate have high concentrations of choline in blood and tissues. In a mouse model, experimentally inhibited uptake and metabolism of choline in embryos was associated with neural tube defects (143). In humans, women in the lowest quartile for daily choline intake had twice the risk of having a baby with a neural tube defect than women in the highest quartile (144). Choline status of the mother in the first half of pregnancy was associated with cognitive development measured with the Bayley Scales of Infant and Toddler Development at 18 months of age (145). In a rat model, supplementation with choline improved memory and learning (146). In rats, there are two sensitive periods when oral choline supplementation gives positive effects on brain function, during neurogenesis and synaptogenesis. Extrapolating to humans, these periods would correspond to a period from in utero to 4 years of age (147). It has been suggested that the effects of choline on neurodevelopment could be due to epigenetic mechanisms (47).

The sphingomyelin concentration of human milk is higher than in formula based on cow’s milk and much higher than in soy-based formula (42). Sphingomyelin and its metabolites ceramide, sphingosine, ceramide-1-P and
Sphingosine-1-P act as second messengers in cell signalling and have regulating effects on apoptosis, cell-cycle arrest, cell survival, cell proliferation and inflammation (148). In a rat model with experimentally inhibited myelination of the nervous system, oral sphingomyelin increased myelination (149). Oral sphingomyelin increased maturation of the intestine in rats (150). Sphingomyelin has also been shown to have cholesterol lowering effects in rats by inhibiting intestinal absorption of cholesterol (151, 152).

Gangliosides are sialic acid-containing glycosphingolipids found in breast milk and known to play a role in neuronal growth, migration and maturation, neuritogenesis, synaptogenesis and myelination (50). MFGM contains gangliosides and ganglioside concentrations in intestinal mucosa and plasma were elevated after MFGM supplementation in a mouse model (153). Supplementation of an infant formula with complex lipids containing gangliosides has in one study, in which the formula was fed from 2-8 weeks until 24 weeks of age, shown increased plasma levels of the gangliosides GM3 and GD3 and a higher Griffith Scales score on hand-eye coordination, performance IQ and general IQ at 6 months compared to a control formula (51). Ceramide, a component of gangliosides, constitutes 10% of the lipid mass of the brain (48).

MFGM has high capacity to carry lipophilic compounds, and therefore MFGM-enrichment has the potential to increase lipid-soluble nutrients in food (154).

The proteome of the human MFGM revealed 191 different identified proteins (155). In a study with bovine MFGM-rich fractions, 244 proteins were identified in a whey protein concentrate and 133 in a buttermilk protein concentrate. Combined, the proteins of the MFGM only represent 1-4% of the total milk protein content, but they have gained much interest because of studies showing that many of them have potential beneficial health effects. Almost 50% of the MFGM-proteins have membrane/protein trafficking or cell signaling functions (156). Fatty acid binding protein (FABP), BRCA1, BRCA2 and beta-glucuronidase inhibitor have proposed anticancer effects, helicobacter pylori-inhibitor prevents gastric diseases and butyrophilin suppresses multiple sclerosis (157). In addition, MFGM has been suggested to have anti-infective properties. Butyrophilin, MUC1, PAS6/7 (lactadherin), CD14, toll-like receptor 1 and 4 and xanthine oxidase are proteins in MFGM with antimicrobial effect (156-159).

Sialic acid is a nine-carbon sugar present in free form or as a structural and functional component of gangliosides, glycoproteins and oligosaccharides.
The oligosaccharide concentration is considerably lower in bovine than human milk (160), and sialic acid in infant formula is mainly bound to glycoproteins (48). The total sialic acid concentration in infant formula is typically <25% of that in breast milk, and the protein-bound/oligosaccharide-bound ratio is 3:1 in infant formula compared to 1:3 in breast milk (44). MUC1 and MUC15 are highly glycosylated proteins with high content of sialic acid that are present in the MFGM (161, 162). Sialic acid concentrations in body fluids reflect metabolic status and body tissue levels. Formula-fed infants have lower concentration of both free and total sialic acid in saliva compared to breast-fed infants (163). The human brain has the highest known concentration of sialic acid in nature, and sialic acid has been suggested as a potential breast milk factor supporting optimal neural function (48). In a pig model, sialic acid supplementation improved memory and learning (49).

Traditionally, during the manufacturing of infant formula, the MFGM fraction has been discarded as vegetable oil instead of milk fat has been used as fat source. With new dairy industry techniques, bovine MFGM-rich fractions are now commercially available and can be supplemented to food (164). MFGM supplementation to complementary food fed from 6 to 11 months of age gave a reduction of diarrhea in a study in Peru (165). MFGM-enriched formula fed to preschool children for 4 months gave a reduction in febrile episodes and behavioural disturbances (166). MFGM supplementation during 4 weeks to adults reduced the trend towards increasing blood lipids concentrations (167).

**Hypotheses of the present trial**

Our main hypotheses were that 1) an experimental infant formula (EF) with reduced energy and protein concentrations would yield a lower energy and protein intake of infants fed EF compared to infants fed standard formula (SF) and a growth closer to that of a breast-fed reference (BFR) group and 2) that supplementation with a bovine MFGM fraction to the EF would enhance neurodevelopment for EF-fed infants. Primary outcomes were weight at 6 months, fat percentage at 4 months and Bayley III-scores at 12 months.

We further hypothesized that the EF group, compared to the SF group, would perform more similar to the BFR group in the secondary outcomes cardiovascular risk markers, infectious morbidity and oral colonization of lactobacilli.
Materials and methods

Subjects

From March 2008 to February 2012, 160 formula-fed infants (80 girls and 80 boys) and a breast-fed reference (BFR) group with 80 infants (40 girls and 40 boys), all born at Umeå University Hospital, Umeå, Sweden, were recruited after inviting parents by telephone. Inclusion criteria were < 2 months of age, gestational age at birth 37-42 weeks, birth weight 2500-4500 g, absence of chronic illness, and exclusive formula-feeding or, for the BFR group, exclusive breastfeeding at inclusion and mother´s intention to breastfeed until 6 months of age, with only small amounts (taste portions) complementary foods, but no formula, allowed between 4 and 6 months. The same recommendations with respect to complementary feeding were given to parents of formula-fed infants. Formula-fed infants were stratified for sex and randomized to receive experimental formula (EF) or standard formula (SF) from inclusion until 6 months of age. Twins were co-randomized to the same intervention group. The intervention was blinded to both parents and staff until all infants had finished the intervention. Powdered formula was distributed to families together with preparation instructions in identical boxes marked with a code number.

Formula composition

BabySemp 1 (Semper AB, Sweden) was used as the SF, and the EF was modified from this formula. Compared to the SF, the EF had reduced energy (60 vs. 66 kcal/100ml) and protein (1.20 vs. 1.27 g/100 ml) densities and was supplemented with a bovine MFGM-enriched whey concentrate (Lacprodan MFGM-10, Arla Foods Ingredients, Viby, Denmark). MFGM proteins constituted 4% (wt/wt) of the total protein content in the EF. The macronutrient contents, fatty acid compositions and amino acid contents of the formulas are shown in detail in Paper I (Tables 1 and 2).
**Measurements and analyses**

Visits were made at baseline (<2 months), 4 months, 6 months and 12 months of age. A summary of measurements at each visit is shown in Table 1. Details of the methods used are presented in respective paper (I-V).

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Baseline</th>
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<th>6 mo</th>
<th>12 mo</th>
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<td>●</td>
<td>●</td>
<td>●</td>
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<td>●</td>
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<td>I</td>
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<tr>
<td>Plasma amino acids</td>
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<td>●</td>
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<td>IV</td>
</tr>
<tr>
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<tr>
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<tr>
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<tr>
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<td></td>
<td></td>
<td></td>
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<td>III</td>
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<tr>
<td>Oral microbial sampling</td>
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*Table 1. Analyses in the study presented by age at measurement.*
**Ethics**

This study was approved by the Regional Ethical Review Board, Umeå, Sweden. During inclusion, which was made by telephone with parents of newly born infants, we were careful not to influence any breast-feeding negatively. This was ascertained by first asking the parent an open question about whether the infant was breast-fed or formula-fed. Parents that had already totally stopped breast-feeding were asked to participate in the randomized part of the study and those exclusively breast-feeding were asked to participate in the BFR group.

**Statistics**

A pre-study power analysis showed that a sample size of 63 in each group was needed to detect a difference of 0.5 SD in the primary outcomes with 80% power at the significance level of 0.05. Expected drop-out rate was 25%, hence 80 infants was included in each group. Most analyses are presented on an intention-to-treat basis, i.e. infants that stopped the intended intervention or breast-feeding were kept in the study and included in analyses. Statistical calculations were made using IBM SPSS Statistics Version 19 (©IBM 1989, 2010) and methods are described in the respective paper (I-V).

The sample size according to the power analysis above was chosen to have a low risk (5%) of a type 1 error, i.e. find a difference when no difference exists. The risk of a type 2 error, i.e. not to find a true difference, is 20%, which is important when interpreting the results. A lack of difference is more uncertain than any found difference.

The randomized design is powerful when comparing the EF and SF groups since background variables are likely to be equally distributed between the groups. Some comparisons between the formula groups are adjusted for relevant co-variates to further ensure a causal relationship between the intervention and outcome.

All comparisons with the BFR group are observational and as always in observational studies, there is a risk of differences in background factors not adequately corrected for. We chose to collect background information to ensure to have enough information to allow comparisons in the randomized design. Many of the comparisons with the BFR group are unadjusted
because we did not have enough background information for an adequate adjustment. Other comparisons are adjusted for relevant co-variates.
Summary of results

Growth, macronutrient intakes and parental control of feeding (Papers I-II)

In a longitudinal analysis, the EF and SF groups did not differ significantly in any of the growth parameter z-scores (weight, length, BMI or head circumference). However, the BFR group differed from the combined formula-fed (EF+SF) groups. The BFR group had higher ΔSDS for weight and length between baseline and 6 months of age. In a cross-sectional analysis, there were no differences between the EF and SF group in any of the growth parameters (weight, length, BMI and head circumference) at baseline, 4, 6 or 12 months of age. The BFR group had a higher length at baseline compared to the formula-fed (EF+SF) groups. There were no differences between the EF, SF or BFR groups in body fat percentage at baseline or 4 months of age as assessed by plethysmography (PeaPod).

Mean daily intake of study formula was larger in the EF group compared to the SF group, resulting in virtually identical energy and protein intakes from formula in the EF and SF groups. When data on intakes of complementary foods were added, there were still no significant differences in energy or protein intakes between 4-6 months of age between the formula groups. The difference in mean daily intake was attributed to larger mean formula meal sizes between 2 and 6 months of age for the EF group. Meal frequency did not differ significantly between the EF and SF groups.

There were no differences in fasting plasma insulin, fasting plasma glucose or blood urea nitrogen (BUN) between the EF and SF groups, but compared to the BFR group, the combined formula-fed (EF+SF) groups had higher plasma insulin and BUN concentrations indicative of higher protein intake.

Parental pressure-to-eat score 12 months postpartum was lower in the combined formula-fed (EF+SF) groups than in the BFR group. After dichotomizing parental control scores to high and low, both high restrictive score and high pressure-to-eat score at 12 months postpartum was associated with infant growth parameters but not with feeding mode.
Cognitive, motor and verbal scores (Paper I)

Cognitive score was 4.0 (95% confidence interval: 1.1-7.0) points higher in the EF than in the SF group. No other significant differences between the EF and SF groups were found in the Bayley-III scales. The BFR group performed significantly better in the cognitive domain compared to the SF group, but not compared to the EF group. Further, the BFR group performed better in the receptive verbal subscale compared to both formula-fed (EF+SF) groups, even after adjustment for psychosocial background variables.

Figure 2. Cognitive, motor and verbal scores at 12 months of age, measured with the Bayley Scales of Infant and Toddler Development, Third Edition, for the experimental formula (EF), standard formula (SF) and breast-fed reference (BFR) groups.

Infections and pneumococcal antibodies (Paper III)

During the intervention, from inclusion until 6 months of age, parents of infants fed the EF reported fewer episodes of acute otitis media (AOM) treated with antibiotics compared to parents of infants fed the SF. The total number of episodes of bacterial infections leading to antibiotics and viral
infections leading to hospitalization during the intervention was also lower in the EF than the SF group. There were no significant differences between the formula groups in other specific bacterial infections treated with antibiotics or viral infections leading to hospitalization before 6 months of age, nor between 6 and 12 months of age.

Figure 3. Proportion of infants in the experimental formula (EF), standard formula (SF) and breast-fed reference (BFR) groups whose parents reported bacterial infections leading to antibiotic treatment or viral infections leading to hospitalization.

Further, the EF group reported significantly less antipyretics use during intervention compared to the SF group. Serum concentrations of immunoglobulins against pneumococci differed between the EF and SF groups. The concentrations of IgG against serotypes ST1, ST5 and ST14 were significantly lower in the EF than the SF group.
Figure 4. Longitudinal prevalence of antipyretics use for the infants in the experimental formula (EF), standard formula (SF) and breast-fed reference (BFR) groups.

**Blood lipids, adipokines, homocysteine, inflammatory markers and blood pressure (Paper IV)**

The mean total s-cholesterol concentration gradually reached higher levels during the intervention in the EF group compared to the SF group, and did not differ significantly in a cross-sectional analysis between the EF and BFR groups at 6 or 12 months. The LDL cholesterol to HDL cholesterol (LDL: HDL) ratio did not differ significantly between the EF and SF groups during the intervention, but the BFR group had higher LDL: HDL ratio than both the EF and SF groups until 6 months of age. Homocysteine levels were lower in the EF group compared to the SF group during intervention. Adipokines and hsCRP-levels were similar in all three groups. Fecal calprotectin was significantly higher in the BFR group compared to the formula-fed groups at baseline. Blood pressure did not differ between the three groups.

**Gastrointestinal symptoms (Paper III)**

There were no significant differences in the frequency of hard stools, abdominal pain, vomiting or consumption of laxatives or probiotic drops between the EF and SF groups. The BFR group had lower frequency of hard
stools, higher stool frequency and consumed less laxatives compared to the formula-fed (EF+SF) groups.

Oral lactobacilli (Paper V)

*Lactobacilli* were cultured less often from saliva of infants in the EF group (9%) and the SF group (5%) compared to the BFR group (34%), p<0.001. The most prevalent lactobacillus was *L. gasseri* found in 88% of the lactobacilli-positive infants. *In vitro*, *L. gasseri* bound to parotid and submandibular saliva, the proteins salivary gp340 and MUC7, purified MFGM and adhered to epithelial cells. Further, *L. gasseri* inhibited *Streptococcus mutans*, *Streptococcus sobrinus*, *Actinomyces naeslundii*, *Actinomyces oris*, *Candida albicans* and *Fusobacterium nucleatum*. 
Discussion

In the present study we tested our hypotheses that feeding healthy term infants with a novel formula with lower energy and protein concentrations and supplemented with bovine milk fat globule membranes (MFGM) would yield functional outcomes in different areas closer to breast-fed infants when compared to infants fed standard formula, by use of a double-blinded randomized controlled trial.

Energy regulation and growth

Infants fed EF fully compensated for the reduced energy and protein concentrations and showed a growth pattern not different from infants fed SF. This did not confirm our original hypothesis that infants fed EF would gain less weight due to a lower energy and protein intake. Previous studies have shown that bottle-feeding is associated with poor self-regulation of intake (22, 24-26, 168-170). Our findings indicate, on the contrary, that bottle-fed infants may have very precise energy regulation in the range from 60 to 66 kcal/100 ml (the range of breast milk) by adopting mean meal sizes throughout the intervention. Further, parents of the formula-fed infants had a low level of parental control of feeding during the first year, and even a significantly lower pressure-to-eat score at 12 months postpartum than parents of breast-fed infants. High parental pressure-to-eat and restrictive scores 12 months postpartum were associated with infant growth but not with formula-feeding. This low level of parental control differs from studies in other populations where parents of formula-fed infants reported higher level of parental control than parents of breast-fed infants (24, 171). The low level of parental control in our study population might explain the unexpected high level of self-regulation for the formula-fed infants, and suggests that, depending on study population, changes in formula energy concentration can be totally compensated for. In such a population, a reduced protein: energy ratio is needed to reduce the protein intake.

The EF and SF groups did not differ in any growth parameters until 12 months of age. However, the BFR group had a slower weight and length gain between baseline and 6 months of age compared to the combined formula-fed groups (EF+SF). Body fat percentage did not differ significantly between any of the groups at baseline or 4 months of age. This is well in line with other studies on formulas with a low protein: energy ratio where no or small differences in growth between formula-fed and breast-fed infants were found
Plasma insulin and BUN concentrations were higher in the formula-fed than in the breast-fed infants, indicative of a higher protein intake of formula-fed infants, in support of previous findings (173-175).

**Cognitive development**

We found that infants fed the EF performed better on cognitive testing at 12 months compared to infants fed the SF, and at a level similar to the BFR group. The size of the difference in cognitive score between the EF and SF group of 4.0 (95% CI: 1.1-7.0) points, is in the same range as the calculated difference in cognitive function between normal-birth-weight formula-fed and breastfed infants of 2.66 (95% CI: 2.15-3.17) points after adjusting for socioeconomic factors in the meta-analysis of observational studies by Anderson et al (34). On an individual basis, an improvement of 4.0 points in cognitive score does probably not give any practical benefits, but on a group level this effect size of the intervention is substantial and to our knowledge, this is the first randomized controlled trial that has shown such a large positive effect of any supplementation to infant formula on cognitive function in term infants measured by the Bayley Scales of Infant and Toddler Development test.

The MFGM fraction contains several components that individually have been associated to brain development, and our interpretation is that MFGM-supplementation has enhanced the cognitive development in the formula-fed infants. From the present study, we cannot deduce the exact mechanism. It could be one single responsible factor (e.g. sialic acid (44, 49), gangliosides (43, 50, 51), sphingomyelin (42, 46, 149), choline (42, 145, 147) or cholesterol (45, 52, 53), a combination of several factors, or it could be different limiting factors for different infants explaining the effect size. The EF and the SF were both supplemented with LCPUFAs to the same level, and it is possible that there could be a synergistic effect of MFGM and LCPUFAs on cognitive development.

**Infections**

Our hypothesis, that MFGM supplementation of infant formula would give a reduction in infectious morbidity, was verified by our findings that infants fed EF had a lower incidence of AOM and a reduced number of days with antipyretic medication during the intervention period than infants fed SF.
Several components of MFGM have been described to have an antimicrobial effect. In a proteomic characterization of human MFGM, 191 proteins were identified of which 20% are involved in immune function (155). In bovine MFGM, 120 different proteins were identified of which 4% have immunological effects and 21% unknown effect (156). Further, the lipid fraction of bovine MFGM had antiviral effect in vitro (67), and gangliosides of the MFGM have been suggested to play an important role in the establishment of intestinal microbiota, gut immunity, and consequently, in the defense of infections (176). The mechanism behind the effect on AOM seen in the present study is unclear. The differences between the EF and SF groups in s-IgG concentrations against pneumococcal serotypes 1, 5 and 14 indicates that a possible mechanism for the protective effect against infections is mediated by or reflected in the humoral immune system, a difference previously shown between breast-fed and formula-fed infants (77). Breast-feeding reduces immune responses, limits hyper-responsiveness and promotes tolerance (74, 75). In the present study, factors in MFGM could have an inhibiting effect on the humoral immune system yielding lower pneumococcal s-IgG concentrations after one or two vaccine doses. Another possible explanation could be that anti-microbial factors of the MFGM exert a local effect on the microbiota in the upper airways in line with numerous identified factors of breast milk (69), which might change the humoral immune response. Growing evidence suggests a link between the gut microbiota and the peripheral immune system (177), and anti-microbial and immunostimulating factors from the MFGM could, by affecting the gut microbiota, have a general effect on the immune system that affects s-IgG concentrations against pneumococci.

**Early metabolic programming**

The present study shows that infants fed the EF differ in serum total cholesterol (s-Ch) concentrations compared to infants fed the SF. By increasing the cholesterol content of the EF, we achieved higher s-Ch, gradually reaching the level of breast-fed infants during the intervention. Theoretically, this could lead to a programming effect and change the cholesterol trajectory for the EF group closer to the BFR group. Breast-fed infants have, compared to formula-fed infants, higher cholesterol levels in infancy, a difference that disappears during childhood to result in lower levels in adolescence and adulthood (137).

However, it is noteworthy that the LDL: HDL ratio did not follow that of the BFR group. In fact, the LDL: HDL ratio did not differ significantly between
the EF and SF group, and the BFR group had higher LDL: HDL ratio from baseline until 6 months of age. This contrasts to the findings in a previous study, with a smaller sample size, where infants fed a formula with high cholesterol content showed elevated total cholesterol at 4 months of age, but also a trend towards elevated LDL: HDL ratio compared to infants fed a low cholesterol formula (178). The impact of early LDL: HDL ratio on any programming effect affecting later cholesterol levels has not been studied in infants, and a long term follow up until adolescence would be desirable.

**Oral lactobacilli**

Oral *lactobacilli* were more often found in the BFR group than the EF and SF groups, and there was no significant difference between the EF and SF groups. It should be noted that cultures were taken from a subsample in the study (EF: n=47, SF: n=43 and BFR: n=43), so the study could be underpowered for detecting a difference between the EF and SF groups. Our results contribute to the field of microbiota and health. The colonization of the gastrointestinal tract from the oral cavity to colon is known to be influenced by different environmental factors including nutrition (124). The interpersonal variability of bacterial community composition is high while the intrapersonal variability is low (179), and the intestinal microbiota has been suggested to contribute to health parameters in different areas, e.g. obesity, atopic diseases, inflammatory bowel disease and intestinal cancers (121). The health consequences of differences in the oral microbiota are less studied. *Lactobacilli* are often found in caries lesions (180) but also seem to have protective effect against caries (181). Systemic health effects could be mediated by nitrate reducing oral bacteria that influence the concentration of circulating nitric oxide involved in vasodilation, nerve transmission, host defence and cellular energetics (182).

**Limitations of the study**

Some differences between the formula-fed groups and the BFR group, e.g. insulin and BUN levels, were seen already at baseline. This is probably caused by the fact that all infants in the EF and SF groups had a period of formula feeding before inclusion. As exclusive formula-feeding was an inclusion criterion, which was formulated to avoid affecting duration of partial breast-feeding in a negative way, only parents of infants that already were exclusively formula-fed were asked to participate in the study. Most parents in all three study groups chose to introduce complementary foods in
the interval 4-6 months. This dilution of the intervention is a weakness of the study leading to an underestimation of the biological effect of the nutritional intervention, but, together with calculations on an intention-to-treat basis, gives a picture of the effect in a population.

Another limitation of the study is the double intervention with both lowered protein/energy content and supplementation with MFGM, with two primary outcomes. The study was designed this way for economical and resource reasons. We predicted that any effect on growth would be caused by the changed energy and protein contents, and that any effect on neurodevelopment or infections would be caused by the MFGM supplementation. As the EF group up-regulated their ingested volumes resulting in similar energy and protein intakes for both formula groups, the probability that the energy or protein intervention had an effect on neurodevelopmental outcomes or infections is low. We cannot, however, exclude an effect of the MFGM on growth or intake.

Due to the study design with randomization between the formula groups, a limited number of background variables were collected, and comparisons with the BFR group should be done with caution. We have chosen to adjust some comparisons for relevant background variables in different multivariate models. Other comparisons were only performed unadjusted when we judged that the collected background variables were insufficient for an adequate adjustment.

**Strengths of the study**

The double-blinded randomized design of the study is a major strength giving high validity to causal relationships of the intervention. All analyses were done on an intention-to-treat basis, i.e. parents of infants that interrupted the intervention before 6 months of age were asked to stay in the study for measurements, giving the true effect of the intervention in a population. The homogenous, high socioeconomic study population is also a strength when assessing the true biological effect of the intervention with little disturbance of background differences.
Conclusions from the present study

We have found a positive effect of MFGM supplementation of infant formula on cognitive function, and this nutritional intervention eradicated the gap in cognitive performance between breast-fed and formula-fed infants at 12 months of age.

Supplementation of infant formula with a bovine MFGM fraction decreased the incidence of AOM in formula-fed infants between 0-6 months of age to a level similar to breast-fed infants, and modulated the concentration of s-IgG antibodies against pneumococci.

Formula-fed infants had the capacity to compensate for a reduction of energy density from 66 to 60 kcal/100 ml by increasing ingested volumes, at least in a population with low parental control of feeding.

Parental control of feeding was influenced by infant growth pattern. An improved formula composition leading to an early growth similar to breast-fed infants might influence not only metabolic programming effects, but also patterns of parental control, and yield beneficial long term effects on growth and metabolic status.

MFGM supplementation of infant formula might influence the higher long-term risk of cardiovascular disease in formula-fed infants by intervening with mechanisms of early programming. We have shown that increasing cholesterol intake between 2 and 6 months of age leads to s-Ch similar to breast-fed infants, but does not change the LDL: HDL ratio towards the level of breast-fed infants.

MFGM supplementation of infant formula did not significantly change the presence of lactobacilli in saliva of formula-fed infants, but lactobacilli were more often present in breast-fed infants. Possible health effects of this are unknown.

The results of the present study are of importance for future improvements of infant formulas that should be based on differences in performance between formula-fed and breast-fed infants (1). We found that supplementation with MFGM narrowed the gap between formula-fed and breast-fed infants in cognitive development and infectious morbidity, but the results need to be confirmed by other studies. Only one dose of MFGM was tested. If the effects would be more pronounced with a higher concentration of MFGM remains to be studied.
A long-term follow-up of the present study is important. A new assessment of cognitive function close to school-age is needed to find out if the difference between the formula groups persists with age. Further, a long time follow-up of growth and metabolic status during childhood, adolescence and adulthood would further elucidate possible programming effects of this nutritional intervention.

**Future work on improvement of infant formulas**

The results of the present study suggest that the MFGM fraction might be responsible for some or all of the observed differences in cognitive development and infectious morbidity between formula-fed and breast-fed infants. Suggestions for future improvements of infant formulas include:

- To further decrease differences in growth between formula-fed and breast-fed infants by lowering the protein: energy ratio. The high protein intake of formula-fed infants is still the most striking difference in macronutrient intakes between formula-fed and breast-fed infants. The high level of self-regulation of energy intake in the present study indicates that moderate changes in energy density probably have little or no effect on energy intake, at least in some populations. Staging of infant formulas with different macronutrient content at different ages during the first 12 months of life would be more physiological and could help to lower the protein: energy ratio, even if this is not allowed according to present regulations (8).

- To test the effect of MFGM supplementation of infant formula in new randomized controlled trials in different settings to support or contradict the findings of the present study, and to test different concentrations of MFGM in infant formulas.

- To test the effects of supplementation with other bioactive milk fractions, preferably in combination with MFGM supplementation to examine additive effects:
  - *Lactoferrin* in human milk has positive effects on iron absorption and has anti-microbial and anti-inflammatory effects. Rice-expressed recombinant human lactoferrin has been expressed and characterized and has been suggested as a beneficial supplementation to infant formula (183). Lactoferrin supplementation of very low birth-weight premature infants reduced the risk of sepsis (184) and one
double-blinded, placebo-controlled pilot study has shown that bovine lactoferrin supplementation of infant formula fed to term infants gave a reduction in lower respiratory tract infections during the first year and higher hematocrit at 9 months (185).

- **Bile salt-stimulated lipase (BSSL)** from human milk has antimicrobial effect *in vitro* (65). Supplementation of premature infants with recombinant human BSSL improved growth and fat absorption (186).

- Other bioactive components of human milk that are lacking or present in a lower concentration in infant formula do not have convincing evidence of positive health effects for supplementation of formula-fed infants. **Cytokines** may give possible beneficial effects on immunology, inflammation and host defense (187). Transforming growth factor-beta (TGF-β) (188), soluble CD14 (189), IL-10 (190) are examples of cytokines where supplementation in animal models have yielded immunological effects, but there is no support from human studies. Numerous **hormones and growth factors** have been the subject of studies. Insulin (191), IGF-1(192) and epidermal growth factor (EGF) (193) stimulate cell growth and maturation. Leptin, adiponectin, IGF-1, ghrelin, obestatin and resistin are also hormones present in breast milk but probably not in formula and have been suggested as likely or possible hormonal mediators of metabolic programming (194). However, there is still no evidence that supplementation with hormones or growth factors to infants would yield any health benefit. Differences in the development of the intestinal flora between breast-fed and formula-fed infants give room for a potential beneficial supplementation with **pre- or probiotics** to infant formula (195). Human milk contains probiotic bacteria and modulation of the intestinal flora with probiotic bacteria has been shown to affect immune function and enhance defense against pathogens (196). One study has shown that enrichment with *Lactobacillus rhamnosus GG* to infant formula increases weight and height between 0-6 months of age (197). Oral supplementation with prebiotic oligosaccharides to formula-fed infants until 6 months of age gave a reduction of allergic manifestations and infections during the first two years of life indicating an effect on the immune system as well as the maturation of the intestinal flora (198). Many studies have also been
performed with negative results and the total body of evidence on supplementation of infant formula with probiotics and prebiotics is weak (199). Supplementation with nucleotides to infant formula has been shown to give a gut flora more similar to that of breast-fed infants with a high proportion of *Bifidobacteria* (200) and to increase head circumference during the first half-year of life (201), but evidence of health benefits are lacking.

**Possible future areas for MFGM**

**Preterm infants**

The health benefits of breast-feeding are larger for preterm than for term infants (34, 55), and human milk is recommended as enteral feeding for preterm infants (202, 203). However, it is not breast-feeding, but the transplacental transport from the mother’s blood that is the physiological route of delivering nutrients to the fetus. Concentrations of nutrients in the cord blood is less examined than concentrations in breast milk, but the placental regulation of different nutrients is of course of greatest importance for fetal growth and development (204, 205). Factors of MFGM might be needed in higher concentrations in the fetus or premature infant than is delivered by breast milk and MFGM supplementation of breast milk to premature infants could theoretically improve outcomes in different areas beyond the benefit of breast-feeding. Possible areas where breast milk has been shown or suggested to have a positive clinical effect and MFGM theoretically could have an additive effect include necrotizing enterocolitis (203), brain development (34), retinopathy of prematurity (206) and infections (207). Further, fat intake has been shown to be positively associated with head circumference growth in extremely preterm infants (208), and components from MFGM could be involved.

**Acquired brain lesions**

The positive effect of MFGM on cognitive development suggested by our results suggests that MFGM may be used to enhance neurodevelopment in other situations. A number of different dietary factors have been tested for injury recovery after acquired lesions in the central nervous system (209), and MFGM could be of interest as supportive in the healing process after
traumatic, vascular, inflammatory, malignant or iatrogenic injuries in the central nervous system.
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