Abstract
Breastfeeding has been associated with many benefits, both in the short and in the long term. Infants being breastfed generally have less illness and have better cognitive development at 1 year of age than formula-fed infants. Later in life, they have a lower risk of obesity, diabetes and cardiovascular disease. Several components in breast milk may be responsible for these different outcomes, but bioactive proteins/peptides likely play a major role. Some proteins in breast milk are comparatively resistant towards digestion and may therefore exert their functions in the gastrointestinal tract in intact form or as larger fragments. Other milk proteins may be partially digested in the upper small intestine and the resulting peptides may exert functions in the lower small intestine. Lactoferrin, lysozyme and secretory IgA have been found intact in the stool of breastfed infants and are therefore examples of proteins that are resistant against proteolytic degradation in the gut. Together, these proteins serve protective roles against infection and support immune function in the immature infant. α-lactalbumin, β-casein, κ-casein and osteopontin are examples of proteins that are partially digested in the upper small intestine, and the resulting peptides influence functions in the gut. Such functions include stimulation of immune function, mineral and trace element absorption and defense against infection.

Introduction
Breastfed infants are known to have a lower incidence of illness than formula-fed infants. This difference is pronounced in less-developed countries where the burden of disease is high, but it is also noticeable in developed countries.
Further, the duration of illness is shorter in breastfed than in formula-fed infants [1]. There are several components of breast milk that are likely to be responsible for this difference in health outcomes. Among these are the gut microflora, which is different in breast- and formula-fed infants, human milk oligosaccharides, and a variety of bioactive milk proteins (table 1) and peptides. Several recent studies suggest that bioactive milk proteins/peptides may have a paramount role in the protection against infection in infants.

**Table 1. Bioactive proteins in breast milk**

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Assessment of Bioactivities of Human Milk Proteins/Peptides

It is difficult to obtain direct evidence that particular components of breast milk provide bioactivities. Randomized clinical trials (RCTs) require large quantities of these components to be added to infant formula and, with few exceptions, breast milk components associated with improved health outcomes are not commercially available in bulk quantities. The exceptions are human milk proteins that are produced in recombinant form, such as bile salt-stimulated lipase (BSSL) and lactoferrin. These recombinant proteins, however, often have different posttranslational modifications (glycosylation/phosphorylation) and are therefore not identical to their native counterparts, are very expensive and also raise ethical concerns. Therefore, the evidence for bioactivities largely rests on in vitro experiments, animal models (mostly rodents) and RCTs, in which bovine milk proteins (or protein fractions) shown to be similar to human milk proteins were studied. In the latter case, in vitro studies showing bioequivalence should be conducted, i.e. that the bovine milk components provide at least some of the bioactivity(-ies) of their human milk counterparts, even when it is recognized that their primary structures differ. An additional approach to assess the physiological significance of a particular protein is to use knockout mice, in which the gene coding for the protein has been deleted/silenced. By
cross-fostering normal (wild-type) mouse pups to knockout mothers lacking the protein in its milk, the particular significance of the milk-borne protein can be evaluated.

**Digestive Fate of Milk Proteins/Peptides**

Most of the bioactivities of milk proteins/peptides are likely to be exerted in the upper gut, i.e. in the stomach and small intestine. Since the bioactivities are dependent upon the tertiary structure of proteins/peptides, the ability of each protein to maintain at least part of its structure after ingestion of a meal, i.e. its capacity to resist digestion, at least for some time, is critical. There are various ways to assess this capacity, ranging from in vitro models to animal models and studies in human infants. In vitro models include lab-scale ‘static’ models in which the gastric pH and pepsin concentration as well as the duodenal/jejunal pH and pancreatic enzymes are modeled after human infants and larger-scale ‘dynamic’ models (e.g. the TNO model) in which the pH and enzyme concentrations, for example, are gradually changed with time to more closely mimic the physiology of digestion. In animal models (rodents or piglets), diets (breast milk or infant formula) are intubated or fed, and the contents of the stomach and small intestine perfused and analyzed at different time points after a meal. In human infants, gastric or duodenal aspirates may be obtained, but this has mostly been limited to preterm infants in which tubes have already been inserted for medical reasons, making the results less generalizable. Another approach is to analyze remaining proteins/peptides in the stool of breast- or formula-fed infants [2].

**Milk Proteins That Can Act in Intact Form**

*Secretory IgA*

The predominant immunoglobulin in breast milk (>90%) is secretory IgA (sIgA). This protein consists of two molecules of IgA linked together via the J-chain and secretory component, which renders the protein resistant against proteolytic degradation. sIgA was early found in intact form in the stool of breastfed infants [3]. Maternal immunity has been shown to be transferred to her breastfed infant via the so-called enteromammary pathway [4] and the infant thus receives protection against the same pathogens to which his/her mother has been exposed to. Addition of sIgA to infant formula has so far not been possible. The major form of immunoglobulin in cow’s milk is IgG, and this protein is rapidly
digested in the infant. Isolation of immunoglobulins from ‘hyperimmunized’ cows, who have been exposed to particular pathogens, and attempts to isolate the very minor fraction of sIgA in cow’s milk have been tried but without much success.

**Lactoferrin**
The multifunctional protein lactoferrin is a major protein in breast milk, constituting about 15–20% of total protein. Lactoferrin has been shown to have bacteriostatic and bactericidal activity, stimulate cell proliferation and differentiation, modulate immune function, facilitate iron absorption, affect brain development and have anti-inflammatory activity [5]. Several of these bioactivities were difficult to reconcile with a local role for lactoferrin in the gut lumen, but with the finding that lactoferrin can bind to a specific lactoferrin receptor on the epithelial cell, become internalized, bind to the nucleus and act as a transcription factor [6], it became possible to find a mechanistic explanation for the multitude of activities reported in the literature. Lactoferrin has been found in intact form in the feces of breastfed infants in significant quantities [2]. The proportion of lactoferrin in the stool is high in early infancy and decreases as the infant’s digestive capacity matures, but even at 4 months of age there is more lactoferrin on a molar basis than there is iron in breast milk, supporting a role for lactoferrin in delivering iron via its intestinal receptor. A clinical trial on infants fed formula with added bovine lactoferrin at a concentration similar to that in human milk showed an increased iron status (hematocrit) and a reduced prevalence of upper respiratory disease [7]. Colostrum is very high in lactoferrin, suggesting that the biological activity of lactoferrin may be very important during the neonatal period. Studies in preterm infants given bovine lactoferrin supplements have shown a reduction in sepsis and necrotizing enterocolitis [8], supporting this notion.

**Lysozyme**
Lysozyme is a small, compact enzyme, which is present in high concentration in breast milk. It has also been shown to be present in the stool of breastfed infants, thus showing resistance against proteolysis [2]. Lysozyme can degrade the cell membrane of Gram-positive bacteria and, thus, has antibacterial activity. It has also been shown that lysozyme can act in a synergistic manner with lactoferrin and kill Gram-negative bacteria [9]. Lactoferrin can bind lipopolysaccharide, a component of the outer membrane of bacteria, and the affinity is so strong that lactoferrin literally creates holes in the membrane, as visualized by electron microscopy [9]. Once these holes have been formed, lysozyme gets access to the underlying proteoglycan matrix and degrades this, thereby killing the bacteria.
There have been no studies on just lysozyme added to infant formula, but as a proof of contest, we added recombinant human lysozyme and recombinant human lactoferrin to oral rehydration solution (ORS) used to treat children with acute diarrhea [10]. In an RCT, hospitalized children in Peru were given regular WHO ORS, rice-based WHO ORS or rice-based ORS with the two proteins combined. We found a significant reduction in duration of diarrhea, stool volume and relapse rate, showing that these two breast milk proteins can be used to successfully treat diarrhea. Since the study did not include groups with lactoferrin or lysozyme only, it is not possible to ascribe the outcome to either component or the combination of the two.

**Bile Salt-Stimulated Lipase**

It is known that lipid digestion is very efficient in breastfed infants. This is particularly evident in preterm infants and likely due to the presence of a high concentration of BSSL in breast milk [11]. This enzyme has the capacity to hydrolyze fatty acids in the sn2-position, which other lipases involved in lipid digestion cannot. The physiological significance of this enzyme has been demonstrated in a clinical trial on preterm infants [12]. In a crossover design, the infants were fed unpasteurized or pasteurized breast milk, in which the BSSL had been inactivated. Infants fed unpasteurized breast milk had significantly lower fat loss in their stool and better growth than those fed pasteurized breast milk, showing that fat was better utilized. Human BSSL has been produced in recombinant form, and clinical trials in premature infants with this enzyme are in progress.

**Milk Fat Globule Membranes**

Milk-based infant formulas consist of a mixture of whey proteins and caseins, usually in a ratio of 60:40, which is achieved by mixing skim milk powder with whey protein concentrate. By far, the majority of milk proteins with ascribed bioactivities reside in these two protein fractions. There is, however, a small protein fraction consisting of milk fat globule membranes (MFGM), which is lost during dairy processing and, therefore, is not part of infant formula. The MFGM protein fraction was determined to be about 2–4% of total human milk protein [13]. Proteomic analysis shows a great diversity of proteins (>115 proteins identified) and many of them are unique to this fraction [14]. Several proteins earlier detected and described in the MFGM, such as lactadherin, MUC 1, MUC 3 and butyrophilin, have been associated with antibacterial and antiviral activities. For example, lactadherin has been shown to inhibit rotavirus in vitro [15], and the concentration of this protein in breast milk was inversely correlated with the incidence of rotavirus infection in breastfed Mexican infants [16]. As a proof-of-
concept study, we performed an RCT in Peru in which infants were given a supplement of a bovine MFGM fraction or skim milk powder twice daily from 6 to 12 months of age [17]. Since micronutrient deficiencies are common in this age group in Peru, 1 RDA of all micronutrients was included in the supplements. We found a significantly reduced prevalence of diarrhea and, in particular, bloody diarrhea in the infants given the MFGM fraction. It should be recognized that we cannot ascertain that this bioactivity was provided by the proteins in the MFGM fraction as it also contains several lipid components such as gangliosides, sphingomyelin, phospholipids and cholesterol. In a more recent study, we evaluated the effects of MFGM in a younger age group by adding it to the formula fed to infants from 6 weeks to 6 months of age [18]. In this RCT, the group receiving formula with added MFGM was compared to infants fed regular formula, and breastfed infants served as a reference group. In this study, we also found health benefits associated with MFGM supplementation as infants fed the MFGM formula had significantly less infections, particularly otitis media, than infants fed regular formula [19]. They were also prescribed significantly less antipyretics. There were no significant differences for these outcomes between infants fed the MFGM formula and breastfed infants. Since the MFGM fraction contains several components involved in early brain development (gangliosides, sialic acid and sphingomyelin), we also assessed cognitive and motor development at 12 months of age by using the Bailey III test. We found that breastfed infants had significantly better scores for cognitive development than infants fed regular formula, which has been shown in several previous studies, but that infants fed formula with MFGM had virtually identical scores as the breastfed infants. Thus, addition of MFGM to infant formula improved cognitive development. As discussed above for the infectious outcomes, we do not know what component(s) caused these effects, but we find it likely that the proteins may prevent disease, whilst lipid components or bound sialic acid affect brain development.

Proteins That Can Act after Forming Bioactive Peptides

α-Lactalbumin
The most abundant protein in breast milk is α-lactalbumin, which constitutes ~20% of total protein. α-Lactalbumin is relatively easily digested and there is no remnant of this protein in the stool of breastfed infants. However, during the passage of the stomach and upper small intestine, digestion of α-lactalbumin results in the formation of several small peptides, which are likely of transitory nature [20]. These peptides have been shown in vitro to have prebiotic activity, be involved in immune stimulation and to enhance mineral absorption. Few
studies on α-lactalbumin-enriched formulas fed to human infants have studied these outcomes, but a study in infant rhesus monkeys showed enhanced iron and zinc absorption [21], providing some support for a role in mineral absorption.

**β-Casein**
The major casein subunit in breast milk is β-casein, a highly phosphorylated protein. During digestion, casein phosphopeptides (CPPs) are formed, and closely spaced phosphorylated threonine and serine residues in CPPs are known to chelate calcium and keep it in solution. These CPPs have been shown to facilitate calcium uptake in cell and animal models, but studies in human adults found no effect on calcium absorption [22]. There have been no studies to date on CPPs added to infant formula. Other peptides formed from β-casein have opioid activity and may, therefore, function along the gut-brain axis, affecting behaviors such as sleep patterns. Again, there have been no clinical trials in human infants with such peptides.

**κ-Casein**
Another major casein in human milk is κ-casein, a heavily glycosylated (>40% carbohydrate) casein subunit. The structures of the glycans on κ-casein are similar to those on the gastric mucosa and the epithelial lining on the small intestine, and in vitro tests have shown that κ-casein can serve as a decoy and prevent attachment of pathogens to mucosal cells [23], thus preventing infection. The structure of κ-casein contains a very sensitive peptide bond, which is rapidly cleaved during digestion resulting in the formation of a glycomacropeptide (GMP). This GMP has been studied to some extent in human adults, and in one study on human infants, the effects of various GMP proportions in infant formula were evaluated [21]. GMP supplementation increased hematocrit, zinc absorption and plasma zinc, supporting an effect on mineral absorption.

**Osteopontin**
A protein that just recently has become of interest in infant nutrition is osteopontin. This protein is known to be involved in various aspects of immune function [24]. Osteopontin is both glycosylated and phosphorylated at several sites in the molecule [25]. It is relatively sensitive to proteolytic degradation, but larger fragments are formed that can still interact with receptor-like structures in the small intestine. Breast milk contains a high concentration of osteopontin, whereas cow’s milk and infant formula contain about 1/10 of this concentration. We have recently conducted an RCT in infants fed formula with two levels of
bovine osteopontin, 50 and 100% of that of breast milk, and infants fed regular formula from 1 to 6 months of age [26]. Breastfed infants were included as a reference group. Infants fed osteopontin-supplemented formula had a serum cytokine profile more similar to that of breastfed infants and different from infants fed regular formula. In particular, levels of the inflammatory cytokine TNF-α were significantly lower in infants fed the formula with osteopontin and breastfed infants. Further, the group fed the higher level of osteopontin had a significantly higher proportion of T cells than the two other formula-fed groups [27]. Days of illness were lower in infants fed the osteopontin-supplemented formulas compared to infants fed regular formula, but similar to breastfed infants, suggesting a beneficial effect on the immune system and health outcomes.

**Formation/Digestion of Milk Peptides – Physiological Implications**

Preformed milk peptides will be ingested by both breastfed infants and infants fed hydrolyzed infant formulas (fig. 1).
Breastfed Infants
It is well recognized that breast milk contains proteases, among them plasmin, but also that there are protease inhibitors [28]. The balance between proteases and protease inhibitors likely varies among women and during the lactation period. The physiological significance of ‘preformed’ peptides in breast milk is not known, but unless the milk is pumped and stored for some time, the extent of this autoproteolysis is likely limited. This is supported by a recent proteomic study on breast milk peptides [29]; the mean total peptide concentration in preterm and term milk was 17.1 and 11.0 μg/ml, respectively. Considering that the total protein concentration of preterm milk is about 20–30 mg/ml (20,000 μg/ml) and in term milk 8–10 mg/ml (10,000 μg/ml), it is obvious that only a small fraction (~0.1%) of all protein in breast milk is in peptide form.

Infants Fed Hydrolyzed Formula
Extensively hydrolyzed formulas were developed for infants with severe milk allergy. They are effective for treatment and prevention of atopic disease, but have a very bitter taste. Partially hydrolyzed formulas were developed to taste better but also with the hope of preventing allergies. Some studies have shown efficacy of such formulas in the prevention of allergy in infants with a strong family history of allergies, whilst others have not [30]. Few studies, however, have investigated the metabolic consequences of feeding such formulas. Extensively hydrolyzed formulas, by their nature, contain very few bioactive peptides [31]. Partially hydrolyzed formulas, though, contain some bioactive peptides, but many of these are broken down when subjected to digestion in vitro [31]. Further, peptides formed by industrial hydrolysis are different from those formed by physiological digestive enzymes (pepsin or pancreatic enzymes). Thus, bioactive peptides formed during normal digestion are lacking and other peptides with less known bioactivities are present in the gut of infants fed partially hydrolyzed formulas. The effects of such differences on the infant need to be investigated further.

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References


