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Evidence for human milk as a biological system and recommendations for study design—a report from “Breastmilk Ecology: Genesis of Infant Nutrition (BEGIN)” Working Group 4

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A B S T R A C T

Human milk contains all of the essential nutrients required by the infant within a complex matrix that enhances the bioavailability of many of those nutrients. In addition, human milk is a source of bioactive components, living cells and microbes that facilitate the transition to life outside the womb. Our ability to fully appreciate the importance of this matrix relies on the recognition of short- and long-term health benefits and, as highlighted in previous sections of this supplement, its ecology (i.e., interactions among the lactating parent and breastfed infant as well as within the context of the human milk matrix itself). Designing and interpreting studies to address this complexity depends on the availability of new tools and technologies that account for such complexity. Past efforts have often compared human milk to infant formula, which has provided some insight into the bioactivity of human milk, as a whole, or of individual milk components supplemented with formula. However, this experimental approach cannot capture the contributions of the individual components to the human milk ecology, the interaction between these components within the human milk matrix, or the significance of the matrix itself to enhance human milk bioactivity on outcomes of interest. This paper presents approaches to explore human milk as a biological system and the functional implications of that system and its components. Specifically, we discuss study design and data collection considerations and how emerging analytical technologies, bioinformatics, and systems biology approaches could be applied to advance our understanding of this critical aspect of human biology.

Keywords: human milk, infant development, immune, microbiome, systems biology

Abbreviations: BEGIN, Breastmilk Ecology: Genesis of Infant Nutrition; BF, breastfed; bLF, bovine milk LF; CHIL, Canadian Healthy Infant Longitudinal Development Study; CS, Cesarean section; DIAMOND, DHA Intake And Measurement of Neural Development Study; FA, food allergy; FF, formula-fed; 2'FL, 2'-fucosyllactose; FUT2, fucosyltransferase-2; GALT, gut-associated lymphoid tissue; HIC, high-income countries; HMO, human milk oligosaccharide; IBD, inflammatory bowel disease; IGF-1, insulin-like growth factor-1; IQ, intelligence quotient; LF, lactoferrin; MFGM, milk fat globule membrane; LNnT, lacto-N-neotetraose; LC-PUFA, long-chain polyunsaturated fatty acids; LMIC, low- or middle-income countries; LRTI, lower respiratory tract infection; NEC, necrotizing enterocolitis; OPN, osteopontin; PROBIT, Promotion of Breastfeeding Intervention Trial; RCT, randomized controlled trial; 6'SL, 6'-sialyllactose; TGF- β , transforming growth factor β ; WG, working group.

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Introduction

The “Breastmilk Ecology: Genesis of Infant Nutrition (BEGIN)” Project was designed to: 1) examine the ecology of human milk, based on the supposition that human milk represents a complex biological system that interacts with both the internal biology and health of the lactating person, the human milk matrix, and the impact on the breastfed (BF) infant and external (social, behavioral, cultural, and physical) environments (see [Text Box 1](#) for Core Concepts and Terms); 2) explore the functional implications of this ecology for both the biological parent and their infant; and 3) explore ways in which this emerging knowledge can be studied and expanded via a targeted research agenda and translated to support the community’s efforts to ensure safe, efficacious, equitable, and context-specific infant feeding practices in the United States and globally. The matrix of human milk refers to the nutrient and nonnutrient components of foods and their molecular relationships to each other (USDA).

The overarching conceptual framework and description of the Project is presented in the BEGIN Executive Summary, the first of 6 manuscripts of this supplement. The subsequent manuscripts in this supplement present the findings of the individual thematic BEGIN Working Groups (WGs) as a continuum of thought that reflects a larger conceptual view of how we can move this important research and public health agenda forward [1–4]. Specifically, the BEGIN Project was accomplished by forming 5 thematic WGs charged with addressing the following themes: 1) parental factors affecting human milk production and composition; 2) the components of human milk and the interactions of those components within this complex biological system; 3) infant factors affecting the matrix, emphasizing the bidirectional relationships associated with the breastfeeding dyad; 4) the application of existing and new technologies and methodologies to study human milk as a complex biological system; and 5) approaches to translation and implementation of new knowledge to support safe and efficacious infant feeding practices. This paper represents the results of the deliberations of WG 4.

The key concept underlying the BEGIN Project is that human milk exists as a biological system that reflects both internal (lactating parent) and external (infant and environmental) influences [5,6]. To advance our understanding of human milk as a biological system, the BEGIN WGs considered interactions within and between each component of the lactating parent–milk–infant triad [1–4]. However, no studies have attempted to integrate datasets across the triad to determine how factors such as genetics of the lactating parent or the physical/social/behavioral

environment of either the lactating parent and/or infant shape the triadic interactions.

Many challenges exist in delineating the functional implications of human milk as a biological system, including the inherent complexity of human milk, the limited availability of noninvasive techniques suitable for use in infants, and the historical lack of application of systems biology approaches to human milk and lactation research. Studies of human milk bioactivity have largely focused on breastfeeding practices (e.g., longer versus shorter duration of breastfeeding), single human milk components, or a limited number of outcomes. These studies fail to account for the full biological potential of the human milk matrix, as viewed within the context of the “nourish, protect, and communicate” paradigm outlined by WG 2 [2].

The task of BEGIN WG 4 was to identify approaches to integrate the interactions of the lactating parent–human milk–infant triad. WG4 explored how emerging analytical technologies, bioinformatics and systems biology approaches could be used to advance our understanding of this critical aspect of human biology ([Text Box 2](#)).

Section 1: Human Milk and Infant Feeding on Outcomes of Term Infants

In this section, we summarize key findings and limitations of available evidence linking human milk composition and feeding practices to infant outcomes, which were selected based on the potential for long-term programming by dietary intake during early postnatal life (see [Text Box 3](#)). A common approach has been to compare human milk–fed to formula-fed (FF) infants. Although these studies have informed how human milk benefits infant development, it is not known whether the differences in outcomes between BF and FF infants result from nutritional components that are present (e.g., bovine milk proteins) or immunologic factors impacting signaling mechanisms (e.g., bioactive components) being present, absent, or available in different levels in infant formula compared with human milk.

Furthermore, the forms and concentrations of nutrients and the matrix in which they exist differ markedly between human milk and infant formula. Three main experimental approaches have been used to study how variability of one or more components *within the matrix* of human milk composition impacts infant outcomes. First, evaluating dietary interventions to the lactating parent to modify one or more components within human milk. Second, studying triads experiencing environmental conditions that could modify human milk composition. Lastly, investigating

Text Box 1

Core concepts and terms

- In the context of this paper, “ecology” is defined as a complex biological system and its interactions with its environment. In this case, the complex system is human milk composition and its inherent biology, and the environment consists of parental and infant inputs and the influence of their respective internal and external environments.
- With due recognition of the need to be observant of issues of gender identity/neutrality, and to improve precision, to the extent possible, for the purposes of the papers described herein, we will use gender neutral terminology where appropriate (e.g., lactating parent/person, etc.), to reflect the reality that not all who lactate identify as female. The term “lactating parent” respects and recognizes those who may have been born female but do not identify as such as well as other gender-relevant contingencies. In situations where reporting primary data (studies/analyses), we will refer to the population as specified (e.g., “the study evaluated 250 lactating mothers”). Moreover, rather than using terms such as “maternal” or “maternal milk,” we will use the terms such as “birthing parent” throughout the report as appropriate as they accurately reflect the biological nature of the birthing parent–infant dyad.
- “Human milk” refers to milk produced by lactating parents and includes both: 1) breastmilk produced by a parent for their infant and fed directly to infants via the breast or expressed by the lactating parent and then fed to the infant; and 2) donor/banked human milk produced by lactating persons that is either donated to human milk banks or fed to infants other than their own child.

Text Box 2

BEGIN WG 4 report outline

- Section 1 Human milk and infant feeding on outcomes of term infants
- Section 2 Human milk components, alone or in combination on outcomes of term infants
- Section 3 Experimental framework and analytical approaches to study human milk as a complex biological system in term infants

genetic differences in the lactating parent or offspring, for example secretor status and human milk oligosaccharide (HMO) content and composition.

In Section 1, we summarize the evidence regarding human milk feeding and its impact on key functional outcomes (Text Box 3). Growth and physical development are highlighted, as studies have been conducted to examine differences in exposure to human milk (e.g., exclusivity and duration) as well as effects of nutrient supplementation and genetics of the lactating parent on infant growth and body composition. Other functional domains are briefly introduced in the text with key observations, proposed mechanisms of action and limitations summarized in Table 1 [7–90].

Physical growth and body composition

Several recent systematic reviews summarize the evidence linking human milk feeding and growth [91–93]. Most studies reported outcomes on linear or body weight gain compared with reference standards, and a few studies report longitudinal changes in body composition [91,92,94–96]. Limitations of existing evidence on whether or how breastfeeding and human milk affect growth outcomes are highlighted in Text Box 4. In addition, none addressed any of the aspects of the human milk ecology.

Exclusivity and duration of human milk exposure

Current evidence associates longer duration of exclusive or partial breastfeeding with slower infant growth rates in term infants than in mixed feeding or formula feeding in developed countries [93]. A recent systematic review of predominantly prospective observational trials concluded that there was insufficient evidence to determine if duration of exclusive breastfeeding reduced risk for obesity [97]; however, moderate evidence supports that ever vs never breastfeeding, particularly for longer than 6 mo, is associated with a reduced risk of overweight and obesity at ages 2 y and older [91,95]. Considering that observational studies are prone to bias due to confounding, a separate analysis was conducted of studies that used more rigorous study designs, including 4 US observational cohorts that included sibling-pair analysis along with the Promotion of Breastfeeding Intervention Trial (PROBIT) in the Republic of Belarus [97,98]. This analysis found that the association between ever vs. never

Text Box 3

Infant functional outcomes of interest for the study of human milk

- Physical growth and body composition
- Endocrine development
- Neurocognitive development
- Intestinal development and microbiome composition
- Immune system development and tolerance
- Metabolome

breastfeeding and risk of childhood overweight and obesity was not significant [97]. Thus, future longitudinal studies are needed involving siblings to assess associations and to provide heightened confidence in causal findings.

To date, PROBIT is the sole cluster randomized study evaluating the effects on multiple outcomes of a breastfeeding promotion program that led to a longer duration of breastfeeding in the intervention group compared with the control group [98]. In addition, health outcomes through adolescence have been reported. As such, PROBIT can be viewed as a model for future study design and expanded to include broader aspects of the internal and external ecologies described in Section 3. Although the PROBIT trial is commonly referenced for breastfeeding outcomes, some limitations have been noted in which Belarus may not be representative of other countries. These include families having access to good basic health services, 3-year maternity leaves with little use of daycare, 95% breastfeeding initiation rate, a well-educated population, and that non-BF infants were excluded from the trial [99].

Analysis of data from the ongoing Canadian Healthy Infant Longitudinal Development (CHILD) birth cohort study revealed an inverse association between breastfeeding and weight gain velocity/BMI in a dose-dependent manner, which was diminished when human milk was fed from a bottle and when human milk was combined with formula supplementation [74,100]. This study investigated important aspects of how infants are fed but has not fully addressed human milk ecology.

Body composition outcomes with breastfeeding are less well studied [101], and only 1 meta-analysis of 11 studies demonstrated that never being BF was associated with altered body composition in infancy, with higher body fat mass in early infancy and lower body fat mass in the second year of life [91]. Hormone content of human milk (leptin, ghrelin, insulin-like growth factor-I [IGF-I], adiponectin, and insulin) has been explored in relationship to infant hunger, fat deposition, and adipose tissue metabolism [102]. To date, no consistent effects were observed, which may be attributed to the reliance on gross anthropometric measures and cross-sectional studies of short duration that did not include early time points. Moreover, traditional component-based studies have not recognized the potential importance of integrating components of human milk while considering its matrix and ecology.

Dietary interventions to the lactating parent

A meta-analysis of data from low- and middle-income countries reported that supplementation with multiple micronutrients during lactation did not improve infant length, stunting, or head circumference [103]. Similarly, a Cochrane systematic review found that long-chain polyunsaturated fatty acid (LC-PUFA) supplementation to lactating parents in high-income countries (HIC) yielded no differences in children's growth [13]. The absence of associations raises questions about the type of supplemented nutrients, their concentration, and the dynamic human milk ecology that may have influenced human milk volume intake [104,105]. More comprehensive study designs accounting for the complexity of human milk composition and its matrix, human milk volume intake and precise longitudinal outcomes could enhance our understanding of the associations between maternal dietary intake and infant development.

Genetic differences affecting human milk composition

The most prominent example of the examination of genetic variations on human milk composition have focused on variation in genes for encoding for secretor and Lewis phenotypes influencing HMO content and composition [106]. Associations between HMO content

TABLE 1
Multifunctional effects of human milk on infant and child outcomes.

Functional System		Findings [reference]
Neurocognitive Development	Observations	<ul style="list-style-type: none"> • A meta-analysis of studies involving >12,000 children reported that those breastfed (BF) for ≤ 6 mos versus >6 mos had 1.04- and 1.06-fold higher scores on intelligence tests than those never BF, respectively [7]. • Associations between variable concentrations of LC-PUFA (8–11) and choline and lutein [12] in human milk and IQ have been shown. • Synergistic associations of higher levels of both choline and DHA in human milk with better recognition memory in infants [12]. • Meta-analyses of RCT of LC-PUFA supplementation have not shown an impact of consumption of fish oil or DHA/EPA supplements in breastfeeding females on cognitive performance of their children [13, 14]
	Proposed Mechanisms or Associations with Human Milk Components	<ul style="list-style-type: none"> • Improved myelination by 2 y of age in BF children, including networks associated with a broad array of cognitive and behavioral skills [15] • Sialylated molecules, including gangliosides and sialoproteins, are present in the frontal cortex of infants [16] • In BF, but not in FF infants, ganglioside-bound sialic acid was correlated with ganglioside ceramide DHA and total n-3 fatty acid, suggesting potential interactions between human milk LC-PUFA and HMOs. [16]
	Limitations	<ul style="list-style-type: none"> • Small sample size studies, short-term studies with short follow-up. lack of diversity, lack of information on maternal and child genetic polymorphisms in LC-PUFA synthesis, lack of report of sociodemographic factors [13,14,17]. • Within human milk component interactions have not been explored (HMO to other components, HMO-HMO interactions).
Endocrine Development	Observations	<ul style="list-style-type: none"> • <u>Type 2 Diabetes</u>: BF reduced risk by 33% [18]. • <u>Type 1 Diabetes</u>: <ul style="list-style-type: none"> • Exclusive BF for >2 wk was associated with a 14% lower risk than in a shorter duration and/or a lack of BF [19–21].
	Proposed Mechanisms or Associations with Human Milk Components	<ul style="list-style-type: none"> • Breastfeeding is associated with lower preprandial serum glucose and insulin concentrations than in formula-feeding [22]. • <u>Type 2 Diabetes</u>: Postulated effects on appetite regulation, reduced weight gain during infancy, and/or nutrients in human milk that promote energy balance, independently of child or adult BMI [23]. • <u>Type 1 Diabetes</u>: <ul style="list-style-type: none"> • Lower gut permeability in BF than FF infants [24] • Immunomodulatory substances, such as lactoferrin, lysozyme, and secretory immunoglobulin A (sIgA), as well as macrophages that affect the function of T- and B cells [25] • Other human milk components have been suggested, but mechanistic evidence in humans is lacking: SCFA [26,27] human milk-derived opioid peptides β-casomorphins [28], HMOs [29], fucosyltransferase-2 nonsecretor status [30], miRNA content of human milk exosomes [31]
	Limitations	<ul style="list-style-type: none"> • <u>Type 1 Diabetes</u>: Unknown whether human milk offers protection in high-risk populations with genetic predisposition [32] • Lack of well controlled studies with longitudinal outcomes and comprehensive information on human milk ecology and composition
Intestinal Development	Observations	<ul style="list-style-type: none"> • Endoscopic biopsies from healthy infants demonstrated 30% greater crypt length in FF than BF infants [33]. • Intestinal permeability <i>in vivo</i> was higher in FF than BF infants [24,34].
	Proposed Mechanisms or Associations with Human Milk Components	<ul style="list-style-type: none"> • Pathways regulating stem cell proliferation, differentiation, and migration, as well as barrier function and immune response were differentially expressed in exfoliated epithelial cells of BF vs. FF infants [35]. • Diet can affect, via gut colonization and cross talk with host epithelial cell, expression of genes associated with the innate immune system in infants [36].
	Limitations	<ul style="list-style-type: none"> • Limited sample sizes (4-10 infants per group) • Lack of longitudinal data on diet and infant intestinal development • Need to delineate developmental from human milk effects (e.g., term vs. preterm) • Need to correlate gene expression with function and developmental outcomes
Immune Development	Observations	<p><u>Immune cells</u>:</p> <ul style="list-style-type: none"> • <u>CD4+ T cells</u>: No consensus for an effect of BF [37–39] • <u>CD8+ T cells</u>: Longer BF was associated with increased CD8 T cell memory, but not memory B cell numbers, in the first 6 mo [39]. • <u>Natural killer cells</u>: Higher in BF than formula-fed infants at 6 mo of age [37]. • <u>Tregs</u>: Proportion of Tregs increased nearly two-fold between birth and 3 wk of age in BF then FF infants [40]. • <u>IgA and IgG secreting B cells</u>: Higher in FF than BF infants [41]. <p><u>Cytokines</u></p> <ul style="list-style-type: none"> • Serum proinflammatory TNF-α and IL-2 were higher, while TGF-β2 was lower, in FF than BF infants in the first year [42].

(continued on next page)

TABLE 1 (continued)

Functional System	Findings [reference]
	<ul style="list-style-type: none"> • BF neonates showed a specific and Treg-dependent reduction in proliferative T-cell responses to noninherited maternal antigens, associated with a reduction in inflammatory cytokine production [40].
	<p><u>IgA:</u></p> <ul style="list-style-type: none"> • Earlier and greater IgA production in nasal and saliva samples from BF compared with FF infants in the first few days of life [43], but not at 7 wks [44]. • Inconsistent finding on salivary IgA, IgM, and IgG between 3 and 6 mos [45,46]. <p><u>Thymic size and GALT:</u></p> <ul style="list-style-type: none"> • Breastfeeding increases thymic size compared with formula-feeding [47]. There are no human studies on the effect of breastfeeding or breast milk on the development of GALT due to lack of access to human tissues.
Proposed Mechanisms or Associations with Human Milk Components	<ul style="list-style-type: none"> • Higher IgA and IgG secreting B cells in FF possibly due to higher antigenic exposure [41]. • Human milk TGFβ may induce IgA production in infants [48]. • IgA production may be induced by <i>Bifidobacterium</i> species (enriched in BF) [19,49]. • Human milk IL-7 content, an important factor for lymphocyte development, was correlated with thymic size [50,51]. • Thymic size was also correlated with the number of CD8+ T cells, which are increased in BF infants [39].
Limitations	<ul style="list-style-type: none"> • Only observational studies. • Small sample sizes and limitations in the detection of memory T cells. • Unknown if mechanisms underlying higher proinflammatory cytokines in formula-fed infants are direct effects of human milk components or are mediated through gut microbiota. • Discrepancies in salivary IgA levels may depend on antigenic exposure and infant gut microbiome composition. • There are no human infant studies on the effect of BF or human milk on the development of GALT, due to lack of access to such human tissues.
Clinical Immune Outcomes	<p>Observations</p> <p><u>Food Allergy:</u></p> <ul style="list-style-type: none"> • Protective effect of exclusive breastfeeding against cow's milk allergy in early childhood among <i>high-risk</i> infants is inconsistent [52,53]. <p><u>Other Atopic Diseases:</u></p> <ul style="list-style-type: none"> • Moderate evidence for a protective effect of human milk consumption against asthma in childhood, limited evidence to indicate no association between human milk consumption and atopic dermatitis in childhood, and inconclusive evidence to suggest a relationship between human milk consumption and atopic dermatitis from 0-24 mo of age [20]. • No association between duration of human milk consumption and allergic rhinitis in childhood [20]. • Evidence is insufficient to suggest any relationships between human milk consumption and asthma, atopic dermatitis, or allergic rhinitis during adolescence or adulthood, or between human milk consumption and food allergy at any life stage [53]. <p><u>Gut Inflammatory Diseases:</u></p> <ul style="list-style-type: none"> • Inconclusive evidence of human milk consumption on celiac disease [54]. • Limited, but consistent, case-control evidence suggests that shorter versus longer durations of any human milk feeding are associated with higher risk of IBD [20].
Proposed Mechanisms or Associations with Human Milk Components	<ul style="list-style-type: none"> • Higher concentrations of total IgA and casein-specific IgA in human milk have been associated with protection against cow's milk allergy [55,56]. • TGFβ is the most well-studied human milk cytokine in connection with infant atopic outcomes; however, a meta-analysis found no association between TGFβ in human milk and allergic outcomes [57]. • High levels of some human milk cytokines (e.g., IL-1β, IL-6, IL-10, and TGFβ) are associated with protection against food allergic disease [58], and human milk IL-6 and IGF-I may play a role in oral tolerance [59]. • Effects of HMO are inconsistent with regard to atopic eczema, cow's milk allergy, asthma and eczema [60–62].
Limitations	<ul style="list-style-type: none"> • High heterogeneity and low-quality evidence, lack of adequate statistical power to assess the impact of BF [53].
Serum and Fecal Metabolomes	<p>Observations</p> <ul style="list-style-type: none"> • BF infants have higher fatty acid metabolism compared with formula-fed infants, as shown by higher levels of free fatty acids, lysophosphatidylcholines, and long-chain acylcarnitines, as well as increased markers of β-oxidation in serum [63]. • FF infants have higher levels of circulating amino acids and amino acid degradation products in serum than BF [64–72]. • Higher levels of microbial degradation products of protein in feces of FF than BF infants [63,66,70,73]. • BF infants introduced to complementary feeding before 6 mos had higher serum BCAA at 12 mo of age than to BF infants who were exclusively BF to 6 mo of age [63,64]. <p>Proposed Mechanisms or Associations with Human Milk Components</p> <ul style="list-style-type: none"> • Differences in dietary composition between human milk and formula • Nutrients in human milk are packaged differently within the human milk matrix (e.g., fats with the MFGM)

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TABLE 1 (continued)

Functional System		Findings [reference]
Gut Microbiome	Limitations	<ul style="list-style-type: none"> • Further studies on human milk composition, including the human milk matrix, and nutrient absorption could determine if changes in human milk composition over time, or even during the day, alter the degree to which human milk nutrients are metabolized and absorbed • Few studies have examined the timing of the introduction of complementary feeding on metabolism • Limited evidence on interactions between diet, microbiome and metabolome and outcomes, such as growth
	Observations	<ul style="list-style-type: none"> • Gut microbiota at 12 mos differed compared with those weaned from human milk before 6 mo of age [74]. • BF infants supplemented with formula where BF was associated with higher <i>B. breve</i> and <i>B. bifidum</i>; cessation of BF resulted in faster maturation of the gut microbiome (Firmicutes) [75]. • Richness and diversity of microbiota were highest in infants who were not BF, lower in partially BF infants, and lowest in exclusively BF infants. Increasing exclusivity of breastfeeding was associated with greater relative abundance of <i>Bifidobacteriaceae</i> and <i>Enterobacteriaceae</i> and lower relative abundance of <i>Lachnospiraceae</i>, <i>Veillonellaceae</i>, and <i>Ruminococcaceae</i> [74].
	Proposed Mechanisms or Associations with Human Milk Components	<ul style="list-style-type: none"> • Glycans cleaved from human milk proteins by microbial glycohydrolases [76] and peptides produced by proteolytic digestion <i>in vivo</i> [77] are bifidogenic • HMOs act as prebiotics and shape the infant microbiome [78]. • <i>B. longum</i> subsp. <i>infantis</i>, directly internalize intact HMOs by specific transporters and degrade them intracellularly [79], which allows it to outcompete other <i>Bifidobacterium</i> species/strains (<i>B. bifidum</i> and some <i>B. longum</i> strains) that use extracellular glycosidase(s) • <i>Bifidobacterium</i> populations increase more rapidly and are more abundant in infants fed by secretor mothers than those fed by nonsecretor mothers and specific <i>Bifidobacterium</i> strains that can use 2'FL are enriched in the stools of the infants receiving human milk of secretors vs. nonsecretors [80,81,82]. • Fucosylated α1-2 oligosaccharides are degraded by some <i>Bacteroides</i> (particularly <i>B. fragilis</i>) and Akkermansia (<i>A. muciniphila</i> MucT) [83], which are commonly present in the infant gut [75]. • Secretor status interacts with route of delivery; infants born by CS who were fed secretor human milk had a less dysbiotic gut microbiota compared with vaginally-delivered infants than did CS infants who received nonsecretor human milk [84]. • Human milk contains a milk microbiota, which has been implicated in seeding the infant microbiota [85].
Limitations	<ul style="list-style-type: none"> • Data on microbiome composition of body sites besides fecal are limited • Most studies are limited to 16S rRNA analysis, with small sample sizes • Data on Archaea, viruses and fungi in human milk and infant stool are limited • HMO diversity and human milk microbiota composition and metabolic function are influenced by milk composition environmental factors, genetics, geographical location, and other factors [86,87], including differences based on secretor status [85,88,89], which are often not documented. • Low biomass samples, such as human milk may yield spurious results due to environmental contamination • 16s rRNA signatures do not reflect viable human milk microbes [90] • Few studies addressed microbiome function (e.g., metagenomics and metabolomics) and human milk matrix • Studies on BF and host-microbe interactions and long-term outcomes are limited 	

BF, breastfeeding; CS, Cesarean section; 2'FL, 2'-fucosyllactose; FF, formula-feeding; GALT, gut-associated lymphoid tissue; HMO, human milk oligosaccharide; IBD, inflammatory bowel disease; SCFA, short-chain fatty acids; Tregs, T-regulatory cells; TGF β , transforming growth factor-beta.

and growth of BF infants have been inconsistent. Variations in concentrations of 2'-fucosyllactose (2'FL), difucosyllactose, lacto-N-neotetraose (LNnT) and lacto-N-fucopentaose I have been associated with weight velocity, height-for-age Z-scores and body

Text Box 4

Limitations of studies investigating infant feeding practices and growth outcomes

- Heterogeneity in outcomes and definitions of infant feeding practices (e.g., feeding at the breast vs bottle, other human milk source [donor milk], exclusivity)
- Inconsistent reporting of nutrient composition and volume of human milk consumed
- Inconsistent growth outcomes reported (body weight, linear growth, body composition, z-scores, weight velocity), and
- Majority of studies are observational, which may introduce bias

composition in some studies but not others [107,108]. Recently, significant associations between human milk bacteria and HMO intakes and concentrations and infant anthropometry, fat-free mass, and adiposity were shown [109]. When data were stratified based on maternal secretor status, some relationships were found among infants born to secretor vs nonsecretor mothers [109]. While these studies have examined associations with all HMOs in the human milk sample, which is more robust than single component associations, they do not encompass the full human milk ecology and matrix complexity and, thus, the conflicting results should be viewed with caution.

In summary, future prospective cohort studies should incorporate robust study design, with standardized and frequent measurements of growth and body composition, measurement of human milk composition and intake, and documentation of pertinent parental and infant co-variables to uncover underlying mechanisms whereby human milk and/or breastfeeding influence infant growth. The extant data is limited in assessing the impact of exclusivity or duration of human milk

feeding, parental supplementation or that of genetic differences on the human milk matrix.

Neurocognitive and functional development

Neurodevelopment is exquisitely sensitive to early life dietary and environmental exposures and has been an active area of research [110]. Some [111,112], but not all [113,114], observational studies of human milk-fed term infants have reported higher cognitive outcomes than in FF term infants, without associating benefit to any particular component of the human milk matrix (Table 1). Deoni et al [15] found that exclusive breastfeeding for at least 3 mo was associated with improved myelination diffusely throughout the brain by 2 y of age, which persisted through early childhood. In a meta-analysis, breastfeeding compared with formula-feeding was associated with an increased intelligence quotient (IQ) of ~3.5 points in childhood and adolescence and 2.19 points after adjustment for maternal IQ [115]. In terms of breastfeeding duration, a meta-analysis of studies involving >12 000 children showed that those BF for either ≤6 mo or >6 mo had higher scores on intelligence tests than those never BF [7].

The effect of LC-PUFA supplementation in the lactating parent, either as fish oil or as purified forms of DHA, EPA, and/or AA on infant neurocognitive development has been investigated. Meta-analyses of randomized control trials have not shown associations between the consumption of fish oil or DHA/EPA supplements in breastfeeding females and the cognitive performance of their children [13,14]. A systematic review, conducted as part of the 2020 Dietary Guidelines, found there was insufficient evidence to evaluate the effects of omega-3 fatty acid supplementation during pregnancy and/or lactation on other infant developmental outcomes [116]. These reviews identified the need for additional studies with larger and more diverse sample sizes and inclusion of information on maternal and child genetic polymorphisms in LC-PUFA synthesis and sociodemographic factors.

Endocrine development

Few studies have investigated how human milk feeding affects endocrine development, other than for risk of type 1 and type 2 diabetes. Updated systematic reviews and meta-analyses suggest that breastfeeding reduces the odds for the development of type 2 diabetes by 33% [18]. For type 1 diabetes, exclusive breastfeeding for >2 wk reduces risk by 14%, compared with a shorter duration [19,20] and/or a lack of breastfeeding [21]. Potential mechanisms and limitations of the current evidence are summarized in Table 1 and include a lack of assessment of the human milk matrix.

Intestinal development

The intestinal tract is functionally immature and immunologically naïve at birth [116] and undergoes marked structural and functional adaptation in response to feeding [24,117]. The trophic response to human milk exceeds that of formula, suggesting a unique contribution of human milk constituents and importantly, the human milk matrix, are important in this response [118,119]. Most studies on the impact of feeding have been conducted in preclinical animal models [120] or preterm infants [121] due to the availability of tissue samples or aspirates. However, measuring the transcriptome of exfoliated epithelial cells has allowed for noninvasive interrogation of intestinal gene expression in term infants [35], affording a noninvasive approach to longitudinally assess the impact of diet on gut health and function in BF infants. Observations of the effect of human milk on intestinal

development of infants and potential mechanisms and limitations of the current evidence, which include a lack of dissection of the human milk matrix effect, are summarized in Table 1.

Immune development and clinical immune outcomes

The immune system is also immature at birth as evidenced by incomplete physical and chemical barriers, poor innate effector cell function, limited and delayed secretory immunoglobulin A (IgA) production, underdeveloped complement cascade function, and insufficient anti-inflammatory mechanisms of the intestinal and respiratory tracts [122]. As reviewed by Dawod et al [123], human milk contains many immune components that are purported to facilitate the transition of the infant to extrauterine life. Microbial colonization of the intestinal tract in early life also plays a key role in stimulating the development of mucosal immunity and long-term programming of the adaptive immune system [49,124–126]. Surprisingly little is known of how human milk influences the developmental ontogeny of peripheral blood immune cells, cytokines or gut-associated lymphoid tissue in human infants [37–47,127] (Table 1).

The impact of human milk consumption on clinical immune outcomes (e.g., atopic diseases and GI inflammatory diseases) is summarized in Table 1. To date, only PROBIT showed that increased duration and exclusivity of breastfeeding was associated with decreased risk of atopic eczema in the first year of life [97]; however, follow-up at 6.5 y did not support a protective effect of prolonged or exclusive breastfeeding on allergy or asthma [128].

Most of the available evidence describing relationships between shorter or longer periods of exposure to human milk, or naturally occurring low versus high levels of human milk components, and outcomes such as IgE development, humoral immunity, and food allergy is limited to observational studies in humans, which are suitable only for suggesting association, not for determining causation. In addition, the evidence is difficult to interpret due to application of varying definitions of breastfeeding and allergic disease, potential for reverse causality, insufficient power for or lack of reporting of specific atopic disease outcomes, and the potential confounding effect of gene-environment interactions.

Serum and fecal metabolome

Metabolomics has emerged as an important tool to investigate how infant diet (human milk vs. infant formula with or without added bioactive components) impacts serum, fecal and urinary metabolites (Table 1). Human milk-fed infants have higher concentrations of fatty acid metabolites [63] than FF infants, who have higher levels of amino acids and products of amino acid degradation [64–71], suggesting that the composition of human milk enhances fat-based metabolism [64, 69].

Fecal microbiome composition

There are multiple microbial ecologies that influence human health and development. While less is known about several of these microbiomes (oral, epithelial, vaginal etc.), emerging evidence indicates a significant role of infant feeding in the development and health of the gut microbiome. The gut microbiome is established over the first 2 to 3 y of life through sequential phases that are influenced by numerous factors, most notably route of delivery and form of nutrition [129,130]. Microbial colonization of the gastrointestinal tract is essential for programming of infant immune, neurocognitive, and intestinal development [49,

123,131]. The process can be viewed through the lens of ‘seeding, feeding, and weeding’, in which route of delivery provides the first exposure to environmental bacteria, which are subsequently shaped by nutrition and antibiotic exposure, among other environmental factors, including geographical location and household exposures [132,133]. The microbiota of infants born by Cesarean section (CS) who were exclusively BF was more similar to vaginally-delivered infants than FF, CS-delivered infants [134]. Thus, when assessing the impact of human milk on the infant microbiota, it is essential to collect metadata related to both birthing parent and infant exposures.

Large longitudinal cohort studies show that breastfeeding duration and exclusivity influence infant gut microbiome composition and function, with exclusive human milk feeding establishing a less diverse and different microbiota that can be disrupted by formula feeding, particularly in the early postpartum period [74,75]. In addition, HMOs act as prebiotics and shape the infant microbiome [78], thus, the genetics (secretor status) of the lactating parent can influence microbial colonization of the recipient infant by modifying the composition of human milk [80]. Observations of the effect of human milk on infant gut microbiome development of term infants and potential mechanisms and limitations of the current evidence are summarized in Table 1.

Conclusions

Existing clinical and epidemiological evidence support improved health and developmental outcomes for infants fed human milk. To date, there is limited research that has explored the full ecology of human milk including critical aspects of the triadic relationships highlighted by WG 1-3 [1–3], and their importance in fully understanding the impact of human milk on infant health and development. Moreover, most studies have focused on a single outcome (e.g., growth, immune development, microbiome composition) rather than taking a holistic view of human milk as a developmental modulator. Although it is important that we apply a systems biology approach to study human milk as a matrix, it is clear that we also need to consider the systems biology within the recipient infant. For example, there is an ever-growing appreciation of the interactions between the microbiota and the development of other organ systems, including gut, immune, and neurocognition. To gain a better understanding of the functionality of human milk components, alone or in combination, the next section will briefly review findings of randomized controlled trials (RCTs) conducted in human infants.

Section 2. Human Milk Components, Alone or In Combination on Outcomes of Term Infants

In addition to the types of studies reviewed in Section 1 that have explored the impact of human milk on specific functional outcomes, considerable effort has gone into the study of specific constituents of human milk on infant health and development. While these studies have rarely explored the intersection of the human milk ecology and the triad, they have provided some potential avenues for pursuit of a deeper understanding of that intersection. The following brief review explores some examples of milk constituents and the most common approaches that have been used to evaluate their impact on infant health and development.

A common approach to the exploration of the impact of milk constituents has been the use of analogs of human milk bioactives isolated from bovine milk or synthesized to study their physiological functions in preclinical models and human infants. Among the most

studied are HMO, milk fat globule membrane (MFGM), osteopontin (OPN) and LC-PUFA [135]. Summarized in this section and in Table 2 are findings of RCTs conducted in infants that evaluated the bioactivities of these milk components. These case studies highlight the multifunctional activities of individual milk components. Notably these studies explore these relationships outside of the biological matrix of human milk, which should be considered when assessing human milk as a biological system.

Human milk oligosaccharides

Select HMOs tested in RCTs were well-tolerated and supported age-appropriate growth in term infants [136]. Two RCTs investigated either 2'FL alone [137, 138] or 2'FL + LNnT [139,140] (Table 2). Compared with the control formulas, supplemental 2'FL alone modulated circulating and secreted cytokines [138]. Parent-reported frequency of illness and antibiotic use was lower in infants fed formula with 2'FL + LNnT [139]. A shift in the microbiome community types was also observed in infants supplemented with 2'FL + LNnT [140]. A more recent RCT assessed the safety and tolerability of a mixture of 5 HMOs at a total concentration of 5.75 g/L, which confirmed there were no differences in growth or tolerance between infants fed standard formula or formula + 5HMO [141]. While confirming the potential immunomodulatory and bifidogenic actions of these specific HMOs *in vivo*, none of these studies recapitulate the composition or complexity of oligosaccharide structures present in human milk.

Machine learning-based classification tools have been used to investigate mechanistic links between fecal microbiome, metabolome, and gut health markers of infants in the 2'FL + LNnT trial [140] who experienced bronchitis or lower respiratory tract infection (LRTI) than in those who did not [139]. Among the main features that discriminated infants who did not experience any reported bronchitis or LRTI were consumption of HMO-containing formula, higher acetate, fucosylated glycans, and *Bifidobacterium*, as well as lower succinate, butyrate, propionate, and 5-aminovalerate, and *Escherichia*. By univariate analysis, infants experiencing no bronchitis or LRTI showed higher acetate and *B. longum* subsp. *infantis* [124]. Of relevance to the question of how to study the complex biology of human milk, this case study illustrates the potential to apply machine learning to existing data sets to identify predictive biomarkers of infant health outcomes in response to human milk components.

Lactoferrin

Lactoferrin (LF), a multifunctional, iron-binding protein, comprises approximately 15% to 20% of total protein in human milk [142], and is considered to be an important contributor to human milk bioactivity. Human LF stimulates immune function, cell proliferation, and differentiation and has antibacterial and antiviral activities [142–144]. Bovine milk LF (bLF) shares 67% amino acid sequence homology with human LF, has similar bioactivities [143,145], and has been tested in several RCTs (Table 2) [144,146,147]. Some studies have shown modest improvements in iron status and lower incidence of upper respiratory illness [146,148] and diarrhea [148] with bLF supplementation; however, other studies have shown no effects [149,150]. In very low birth weight infants, early studies showed that giving bLF orally decreased necrotizing enterocolitis (NEC), sepsis and mortality [151, 152]. Recent multicenter RCTs have shown no benefit of oral bLF [153,154]. These divergent findings may be due to different sources of bLF, its heat treatment before administration, or contamination by other proteins [155]. Infants fed formula with both bLF and MFGM showed improved neurodevelopmental outcomes [156], but it is not possible to

assess if both components contributed to the improved outcomes as other studies have shown that MFGM alone affects cognitive development. To date no studies have been found that have explored LF from the perspective of the interactions of the triad either in terms of LF composition or impact.

Milk fat globule membrane

Fats in human milk are packaged within a 3-layered membrane, or MFGM, which contains anti-infective proteins, carbohydrates (sialic acid), and lipids (gangliosides and cholesterol), the latter of which are purported to be involved in neurodevelopment [157]. Readers are referred to the WG 2 report [2] for additional information on the composition MFGM and its potential biological roles in signaling and cell membrane composition.

Formula contains very little or no MFGM as defatted bovine milk is used as the starting material. RCT have investigated supplementation of formula with bovine milk MFGM, which is not identical to human milk MFGM, but contains similar components and at similar levels [158,159] (Table 2). The addition of a bovine milk protein isolate enriched in MFGM at 4% of total protein to an infant formula was associated with significantly lower rates of infection, and in particular otitis media, than in formula alone [159,160]. MFGM-supplemented infants had a lower abundance of oral *Moraxella catarrhalis*, a microorganism commonly linked to otitis media, providing a potential mechanism of action [161]. There were minor effects of MFGM on fecal microbiome composition [63]. At 4 and 6 mo of age, both the fecal [63] and serum metabolomes [64,69] and serum and erythrocyte cell membrane lipidomes [162] differed between infants fed formula with MFGM vs formula alone; however, differences were no longer present at 12 mo. Lastly, at 12 mo of age, infants fed MFGM-containing formula had significantly higher scores in the Bayley III cognitive domain than infants fed formula without MFGM [159], but a follow-up study at 6.5 y of age found no differences in neurodevelopment between children who were fed the MFGM-containing formula compared with unsupplemented formula [163]. Further complicating our understanding of the contribution of MFGM to human milk bioactivity is that the composition of bovine MFGM used in RCTs varies considerably among commercial suppliers [157].

As with the previous components discussed in this section, while there is evidence to suggest a functional impact of the MFGM on infant related outcomes, no studies were found that explored the nature of the triadic relationships on MFGM content or functional impact. Furthermore, a need exists to apply the ecological systems approach advocated throughout the BEGIN WG reports to advance our understanding of the biology of the MFGM not just as a component of human milk, but as a unique biological system in and of itself.

Osteopontin

OPN is abundant in human milk, but is low in cow's milk and, consequently, infant formula [164,165]. OPN stimulates cell proliferation and differentiation and exerts immunomodulatory functions by binding to integrin and CD44 receptors on cell membranes [164]. Interestingly, unlike other components reviewed in this section, there is evidence of an important role of the triadic relationship in OPN content and function. For example, factors in the lactating parent such as BMI, birth route, pregnancy weight gain, and energy intake during lactation affect human milk OPN concentrations [166]. Consistent with immunomodulatory activities, negative associations between human milk OPN levels and fever-related infant hospitalizations from 0 to 3 mo of

age and have been reported, suggesting an interaction between the infant and OPN content within the human milk matrix [166].

An RCT studied 1- to 6-month-old infants who were fed standard formula, formula with bovine OPN added at half the human milk level (65 mg/L) or formula with the same level as in human milk (130 mg/L) and compared these groups with a BF group [167]. Infants fed formula + OPN had significantly less morbidity (days with fever), lower proinflammatory and higher anti-inflammatory cytokine levels in serum [167], and differences in circulating lymphocyte subsets [168] than infants fed standard formula. A follow-up analysis reported higher human OPN concentrations in plasma of both formula + OPN groups and BF infants than in infants fed standard formula [165]. In addition, plasma bovine OPN concentration was greater in the 130 mg/L OPN group than the 65 mg/L group [165], suggesting that dietary bovine OPN was absorbed and could affect endogenous OPN synthesis and secretion [165]. A finding that OPN and LF form a strong complex that enhances resistance against digestion and several bioactivities [169] should be considered when evaluating the matrix of human milk.

The evidence of the important immunomodulatory role of OPN and the potential impact of the response of both the parent and infant to infection reveals both an intimate relationship between the triad and OPN content, as well as the potential importance of the compound in our response to current and emerging infectious diseases.

Long-chain polyunsaturated fatty acids

LC-PUFAs are the most studied functional human milk components, with AA, DHA, and EPA being the most commonly studied n-6 LC-PUFAs and n-3 LC-PUFAs, respectively. Over 40 y ago, dietary LC-PUFA intake by the lactating parent was shown to affect human milk. LC-PUFA concentration [170] and human milk LC-PUFA was linked to infant LC-PUFA status [171,172]. At the same time, other investigators showed the importance of DHA for neurodevelopment [173,174] and identified that the period between 24 wk gestation and infancy was critical for brain DHA accumulation [175,176]. At that time, infant formula lacked preformed AA and DHA, precipitating a number of RCTs to study the effects of LC-PUFA supplementation to the lactating parent (see Section 1) or added to formula fed to term or preterm infants. These findings have been reviewed in recent systematic reviews and meta-analyses [177–179] and are briefly summarized in Table 2.

The DHA intake and measurement of neural development (DIAMOND) study is the only dose-response RCT of DHA and AA that investigated the addition of AA (0.64% of total fatty acids) and 4 doses of DHA (0%, 0.32%, 0.64%, and 0.96% DHA) to infant formula, which was fed for the first year of life [180]. In this study, improvements were observed in cortical visual acuity [180] and cognitive outcomes in all DHA groups compared with 0% DHA, some of which persisted through 6-9 y of age (Table 2) [181–183].

However, 2 recent systematic reviews found no benefit of LC-PUFA supplementation on longer-term cognitive outcomes [177, 184]. In the first, cognitive performance of term-born children >2.5 y (range 3.3–16 y), assessed by the Wechsler Preschool and Primary Scale of Intelligence-Revised, showed no effect of early LC-PUFA supplementation. Similarly, performance of preterm-born children on the Wechsler Abbreviated Scale of Intelligence was not affected early LC-PUFA supplementation [177]. The same group of investigators compared differences in academic performance of term-born or preterm adolescents who had participated in one of 7 RCTs of formula supplementation as infants, 2 of which were LC-PUFA supplementation studies [184]. At 11 y of age, preterm and term participants

TABLE 2
Multifunctional bioactivities of isolated milk components supplemented to infant formula.

Milk Component and [concentration] (ref)	Functional Outcome					
	Growth/Tolerance	Infection	Immune Function	Neurocognitive	Microbiome	Serum Metabolites
HMOs						
<ul style="list-style-type: none"> • 2'FL [0.2 or 1.0 g/L] + galacto-oligosaccharides (GOS) to 2.4 g/L • 4 month intervention • BF reference [136,137] 	<ul style="list-style-type: none"> • Growth of 2'-FL supplemented infants not different than SF [136] 	<ul style="list-style-type: none"> • NR 	<ul style="list-style-type: none"> • 2'FL reduced circulating pro-inflammatory cytokine concentrations and their secretion by isolated PBMC stimulated with RSV compared with SF; levels not different than [137] 	<ul style="list-style-type: none"> • NR 	<ul style="list-style-type: none"> • NR 	<ul style="list-style-type: none"> • 2'FL detected in blood and urine of BF infants and those fed formula + 2'-FL [136]
<ul style="list-style-type: none"> • 2'FL [1.0 g/L] and LNnT [0.5 g/L] • 6 month intervention • Follow-up at 12 mos [138,139] 	<ul style="list-style-type: none"> • HMO-supplemented infants had softer stool and fewer nighttime wake-ups at 2 mo of age [138] 	<ul style="list-style-type: none"> • Reduced infectious episodes in the HMO-supplemented group [138] • Fewer parental reports of bronchitis through 4, 6, and 12 mo and LRTI through 12 mo [138] • Reduced parent-reported antipyretics use through 4 mo and antibiotics use through 6 and 12 mo [138] 			<ul style="list-style-type: none"> • Microbial alpha and beta diversity of the HMO group was closer to that of the BF than control formula [139] 	
<ul style="list-style-type: none"> • 5 HMO: LNT, 2'FL, 3'FL, 3'SL, 6'SL • Total HMO concentration of 5.75 g/L • 4 month intervention [141] 	<ul style="list-style-type: none"> • No differences in growth or tolerance between infants fed SF or formula + 5 HMO 					
Lactoferrin						
<ul style="list-style-type: none"> • Term infants fed formula with native bLF [102 mg/L] or added bLF [850 mg/L] for 12 mo [146] • Term infants fed formula or added bLF [38 mg mg/L] for 3 mo (105) 		<ul style="list-style-type: none"> • Reduction of URTI and wheezing (P < 0.05) • Lower respiratory-related illnesses and occurrences of diarrhea-related illnesses BF and bLF groups than CON (P < 0.05) 				<ul style="list-style-type: none"> • Higher hematocrit levels at 9 mos (37.1% vs 35.4%; P < 0.05) in bLF-supplemented than CON
<ul style="list-style-type: none"> • Preterm, VLBW infants • Multicenter, randomized, double-blind, placebo-controlled trial • Orally dosed with bLF [100 mg/d, LF]; LGG [6×10⁹ CFU/d [151] 	<ul style="list-style-type: none"> • No adverse effects or intolerances to treatment occurred [151] 	<ul style="list-style-type: none"> • Incidence of ≥ stage 2 NEC and of death-and/or ≥ stage 2 NEC was lower in LF (p =0.055) and LF+LGG (p<0.001) vs. CON 				

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TABLE 2 (continued)

Milk Component and [concentration] (ref)	Functional Outcome					
	Growth/Tolerance	Infection	Immune Function	Neurocognitive	Microbiome	Serum Metabolites
<ul style="list-style-type: none"> • bLF+LGG]; or placebo (CON) • Birth to 30 d of life [151, 152] • Preterm, VLBW infants <32 wk GA • Multicenter (37 centers), randomized, double-blind, placebo-controlled trial • Orally dosed with bLF [150 mg/kg/d, LF]; or sucrose [CON] • ≤ 72 hours postpartum to 34 wk PMA [153] • Preterm infants weighing 500-2000 g • Multicenter (3 centers), randomized, double-blind, placebo-controlled trial • Orally dosed with bLF [200 mg/kg/d, LF]; or sucrose [CON] • 8 week intervention • Follow-up at 24 mo [154] • LF (0.6 g/L +MFGM (5 g/L) for 1 y study • Follow-up at 1.5 y [156] 	<ul style="list-style-type: none"> • Growth outcomes and rehospitalization rates during the 2-year follow-up were similar in both groups • No difference in growth vs. CON 	<ul style="list-style-type: none"> • Incidence of LOS due to bacterial or fungal infection was lower in LF (p=0.002) and LF+LGG (p<0.001) vs. CON (109) • 316 (29%) of 1093 LF infants acquired a LOS vs 334 (31%) of 1089 CON infants • Risk ratio adjusted for minimization factors was 0.95 (95% CI 0.86-1.04; p=0.233). • LOS or sepsis-associated death occurred in 22 LF infants (10.5%) vs 30 (14.6%) CON • No difference after adjusting for hospital and birth weight; hazard ratio 0.73 (95% CI, 0.42-1.26). • At 24 mo, LF infants had less bronchiolitis than CON (rate ratio, 0.34; 95% CI, 0.14-0.86). • Respiratory-associated adverse events and diarrhea were significantly lower for the MFGM + LF group through 1.5 y (p<0.05) 				
<p>MFGM</p> <ul style="list-style-type: none"> • MFGM protein fraction twice daily added to weaning food • 6- to 11-month-old infants [158] • Formula with or without added 	<ul style="list-style-type: none"> • No effect of MFGM on growth or 	<ul style="list-style-type: none"> • Global prevalence of diarrhea was 3.84% in MFGM vs 4.37% in CON (P < 0.05). • MFGM reduced episodes of bloody diarrhea by 46% (P = 0.025) • Lower incidence of diarrhea, otitis 		<ul style="list-style-type: none"> • Mean age-adjusted normalized Mullen composite score at 24 mo was 83.3 ± 13.6 in the LF group vs. 82.6 ± 13.1 in CON (N.S.). • Bayley II mean cognitive (+8.7), language (+12.3), and motor (+12.6) scores were higher (P< 0.001) for the MFGM + LF group vs. CON at 1 y • Differences no longer present at 1.5 y 		
				<ul style="list-style-type: none"> • At 12 mo of age, Bayley cognitive 	<ul style="list-style-type: none"> • Little effect on fecal 	<ul style="list-style-type: none"> • MFGM reduced fecal lactate,

(continued on next page)

TABLE 2 (continued)

Milk Component and [concentration] (ref)	Functional Outcome					
	Growth/Tolerance	Infection	Immune Function	Neurocognitive	Microbiome	Serum Metabolites
<p>MFGM fed for 4 mos</p> <ul style="list-style-type: none"> MFGM proteins constituted 4% (wt:wt) of the total protein content ~2-6 month-old infants Follow-up at 12 mo BF reference [63, 64, 159–163] 	<p>tolerance vs. CON [169]</p> <ul style="list-style-type: none"> At 6.5 y of age, no differences between MFGM and CON in weight, length, or head or abdominal circumference [163] 	<p>media and anti-pyretic use vs. CON [159, 160]</p>		<p>score was higher (P = 0.008) in the MFGM (105.8 ± 9.2) than CON (101.8 ± 8.0), but was not different than BF (106.4 ± 9.5; P = 0.73) [159].</p> <ul style="list-style-type: none"> At 6.5 y of age, no differences between MFGM and CON in any measure of cognitive or adaptive functioning [163] 	<p>microbiome between MFGM and CON [63]</p> <ul style="list-style-type: none"> MFGM fed infants had a lower abundance of oral <i>Moraxella catarrhalis</i> than CON [161]. 	<p>succinate, amino acids and their derivatives vs. CON [63]</p> <ul style="list-style-type: none"> Infants fed MFGM had higher levels of fatty acid oxidation products in serum than CON [64] Plasma lipidome of infants fed MFGM differed at 4 mo (SM and PCs) and 6 mos (SM, PCs, ceramides) vs. CON [162] Erythrocyte SMs, PEs and PCs differed between MFGM and CON at 6 mo [162] At 6.5 y of age, no differences between MFGM and CON plasma concentrations of homocysteine, lipids, insulin, or glucose [163]
<p>Osteopontin</p> <ul style="list-style-type: none"> Double-blind RCT Formula with 0 (CON), 65 (F65) or 130 (F130) mg/L bovine OPN for 6 mos BF reference [165,167,168] 	<ul style="list-style-type: none"> Growth patterns, formula intake, sleep patterns and adverse events were similar in all formula-fed groups [167] 	<ul style="list-style-type: none"> Infants fed OPN had significantly fewer days with fever than CON [167] 	<ul style="list-style-type: none"> Infants fed OPN had serum TNF-α and higher IL-10 than CON (124) Infants fed F130 had higher T-cell proportions than F0 or F65 [168] 			<ul style="list-style-type: none"> At 4 and 6 mo, plasma human OPN was higher in BF, F65, and F130 than CON [165] Plasma bovine OPN in F130 was greater than F65 [165]
<p>LC-PUFA</p> <ul style="list-style-type: none"> Double-blind RCT (DIAMOND STUDY) Term infants, n=~40 per group Compared 4 formula containing 0.64% AA and either 0% (CON), 0.32%, 0.64% or 0.96% DHA for 12 mo [180] 				<ul style="list-style-type: none"> Any level of DHA compared with CON (0% DHA) improved: Visual acuity in infants fed at 12 mo, but not 3 mo [180] Cognitive development through 6 y of age [181] Brain ERP responses and synchronization during a task requiring response inhibition at 5.5 y [182] Brain structure, function and metabolism at 9 y [183] 		

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TABLE 2 (continued)

Milk Component and [concentration] (ref)	Functional Outcome					
	Growth/Tolerance	Infection	Immune Function	Neurocognitive	Microbiome	Serum Metabolites
<ul style="list-style-type: none"> • RCT • Preterm infants <33 wk GA and 750-1800 g) • Compared CON formula with formula containing 0.26% DHA and 0.42% AA from either fish/fungal or egg/fish oil sources • Body composition measured by DXA [185] • Subset of term infants in the DIAMOND STUDY • Measured growth outcomes from 6 y. • A limitation is the small sample size at 6-year follow-up (n=18-24/group) [186] 	<ul style="list-style-type: none"> • No significant differences among the 3 groups at any time point in weight, length, or head circumference or bone mineral content or density. • Greater (p<0.05) lean mass and reduced fat mass in infants fed formula with LC-PUFA at 12 mo • Compared CON formula, children who consumed LC-PUFA supplemented formula had higher length-/stature- and weight-for-age percentiles, but not BMI percentile from birth to 6 y • Maternal smoking predicted lower stature (2-6 y), higher weight-for-length (birth-18 mos) and BMI percentile (2-6 y) independent of LC-PUFA effects. • Gender interacted with the effect of LC-PUFA on stature, and the relationship between smoking and BMI, with a larger effect for boys. 					

2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 3'FL, 3'-fucosyllactose; 6'SL, 6'-sialyllactose; BF, breastfed; CON, control; GOS, galacto-oligosaccharides; GA, gestational age; HMOs, human milk oligosaccharides; LNnT, lacto-N-neotetraose; LNT, lacto-N-tetraose; LOS, late onset sepsis; LC-PUFA, long-chain PUFA; LRTI, lower respiratory tract infection; MFGM, milk fat globule membrane; NR, not reported; OPN, osteopontin; PC, phosphatidylcholines; PMA, postmenstrual age; RSV, respiratory syncytial virus; SM, sphingomyelins; SF, standard formula; URTI, upper respiratory tract infection.

randomized to LC-PUFA supplemented formula scored lower in English and mathematics than those not supplemented as infants. These observations highlight not only the need for longer-term follow-up of infant feeding studies but also for addressing heterogeneity in neurocognitive assessment measures.

Differences in growth and body composition in response to LC-PUFA supplementation have been reported in preterm and term infants. Preterm infants fed formula with DHA and AA had increased lean mass and reduced fat mass compared with infants fed formula without LC-PUFA [185]. A follow-up of term infants enrolled in the DIAMOND study [180] found increased length- and weight-for-age, but not higher BMI, through 6 y in those fed LC-PUFA as infants [186]. However, both of these studies were small and a recent Cochrane review of 13 studies that measured physical growth of term infants found no beneficial or harmful effects of supplementation [178].

Lastly, the influence of LC-PUFA supplementation on infant immune development has received considerable attention. In some studies, infants fed formulas with both DHA and AA, exhibited less skin and respiratory allergic diseases in childhood compared with those

fed formulas without LC-PUFA [180]. An analysis of infection and allergic outcomes of >8,000 infants enrolled in the ELFE French longitudinal birth cohort reported that consumption of DHA/AA/EPA-enriched formula (especially those with high EPA content) was associated with a lower risk of LRTI and lower use of asthma medications than in infants fed nonenriched formula [187].

The value of systems approach with specific regard to these compounds is that questions remain with regard to need for and value of LC-PUFA at specific times in infant development, e.g., the value of supplementation to preterm vs term infants. A systems approach to the triad would shed light on not only the biology but the metabolism and chronobiology of these compounds to determine the role of parental supply and critical periods of need for the infant.

Conclusions

This limited review of RCTs has provided evidence that individual human milk components, or their bovine analogs, supplemented to infant formula at levels present in human milk affect specific aspects of infant development. However as illustrated in Table 2 and Figure 1, a

single component, such as OPN or MFGM, affects more than one infant outcome (e.g., infection, microbiome, and cognition). These observations reinforce the need to consider interactions among components within the human milk matrix as well as interactions *within the infant* as development of one system in the infant (e.g., microbiome) can impact infant immune, gut, or brain development (Figure 1). For example, HMO may directly affect immune or brain development or may act indirectly via modulation of gut microbial composition or function.

To decipher the complexity of human milk, systems biology approaches, which are based on the understanding that networks that form the whole of living organisms are more than the sum of their parts, will be needed.

Section 3: Experimental Framework and Analytical Approaches to Study Human Milk as a Complex Biological System in Term Infants

The central premise of the BEGIN project is that human milk is a complex biological system. As discussed throughout the BEGIN reports [1–5], human milk composition consists of many interacting components, is embedded within the lactating parent–human milk–infant triad [188] and is affected by communication between the lactating parent and infant. As highlighted by Smilowitz et al. [2], studying a single element of this triad, or a single component of human milk, ignores their integrated nature and limits our ability to understand the determinants and consequences of human milk composition and function.

We propose that the biology of breastfeeding and human milk should be studied as a “system-within-a-system” (Figure 2), which has not been fully undertaken to date. The suggested approach will require careful planning from the outset by transdisciplinary teams of researchers to incorporate extensive biological and metadata collection, advanced analytical approaches, and systems biology integration (Figure 3). As will be described below, Foundations (e.g., the Bill and Melinda Gates Foundation) have undertaken

multicountry breastfeeding interventions in recent years; however, most have not collected data that will enable full interrogation of the lactating parent–human milk–infant triad. The understanding of the importance and the complexity of the triad by funding agencies will enable resources to be directed to more comprehensive studies in the future. The following sections provide descriptions of key elements of this approach.

Essential components of the human milk research infrastructure

Studies intended to expand our understanding of the complex biology of human milk, particularly those involving large subject cohorts, will require attention to a number of key elements of study design and relevant technology. One approach would be the establishment of a longitudinal cohort study specifically designed to collect, analyze, and integrate the complexity of human milk as a biological matrix, including an interrogation of the parental and infant factors influencing that complexity, and the short- and long-term implications of human milk on relevant infant and child health outcomes. In addition, a need exists for resources to collect and annotate existing data and biological samples and to generate guidelines for broader data capture for ongoing or future single- or multiinvestigator studies and interventions, which will be essential for advancing the human milk research agenda (Text Box 5).

In particular, human milk repositories have an important role to play in these efforts. Two examples in North America are the Mommy’s Milk Human Milk Biorepository at the University of California, San Diego [190] and the Manitoba Interdisciplinary Lactation Center [191]. By using standardized protocols for sampling and storage, collecting, and cataloging human milk from large populations with diversity in race and ethnicity, geography, and socio-demographic factors, repositories can serve as a central platform for interdisciplinary human milk research. Individual samples can be aliquoted at the time of processing and then analyzed for multiple components, and/or accessed by multiple investigators with different

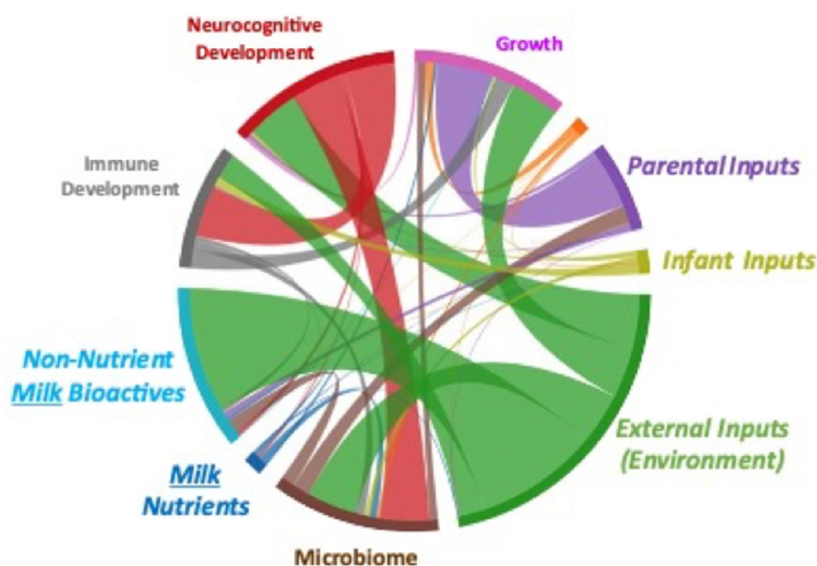


FIGURE 1. Representative chord diagram illustrating multilevel interactions that require systems biology approaches. Systems biology is an approach in biomedical research to understand the larger picture of interactions within the matrix of human milk, on multiple systems within the infant, which also interact (e.g., the microbiome–gut–brain axis).

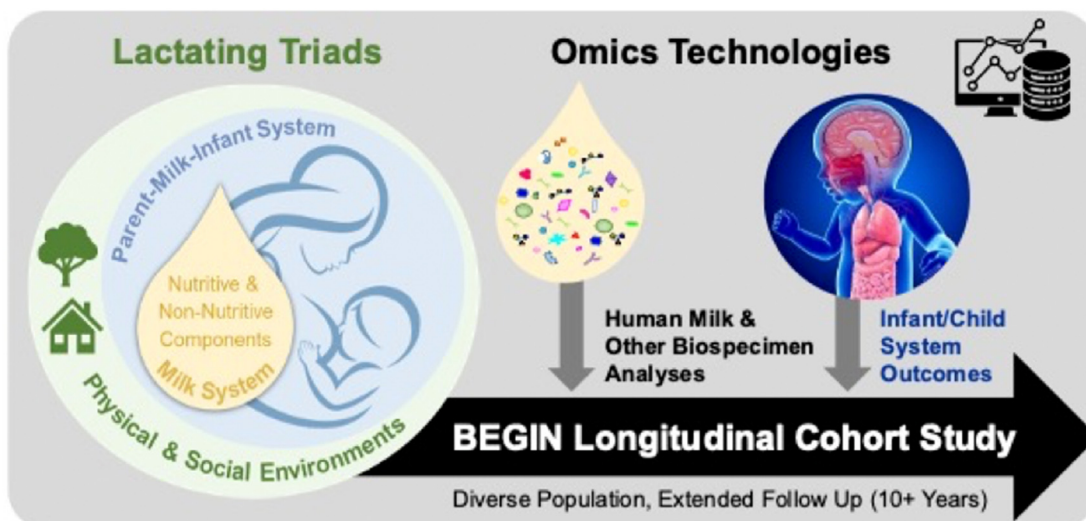


FIGURE 2. Biological effects of human milk as a system-within-a-system. Human milk is a complex biological system comprising many components within a unique matrix that is imbedded within the lactating parent–human milk–infant triad. Human milk composition is shaped by both external and internal ecologies unique to each lactating parent and infant dyad. Human milk influences the development and maturation of interacting systems within the infant (e.g., immune, microbiome, intestinal, brain), which influence long-term outcomes in the infant.

research questions. Repositories that can link human milk samples to electronic health data for lactating parents and infants are especially powerful, offering low-cost opportunities to perform longitudinal studies on the association of human milk components with lactating parent and child health. When multiple datasets are generated using the same human milk samples, and appropriate data sharing agreements are in place, repositories offer exciting synergistic

opportunities to undertake multi-omic analyses on human milk samples from well-characterized triads.

Specific study considerations

As stated by Steven Covey, “Begin with the End in Mind” [192], which entails creating a clear vision of direction and destination to help in attaining a goal. In this case, the five-stage nonlinear translational

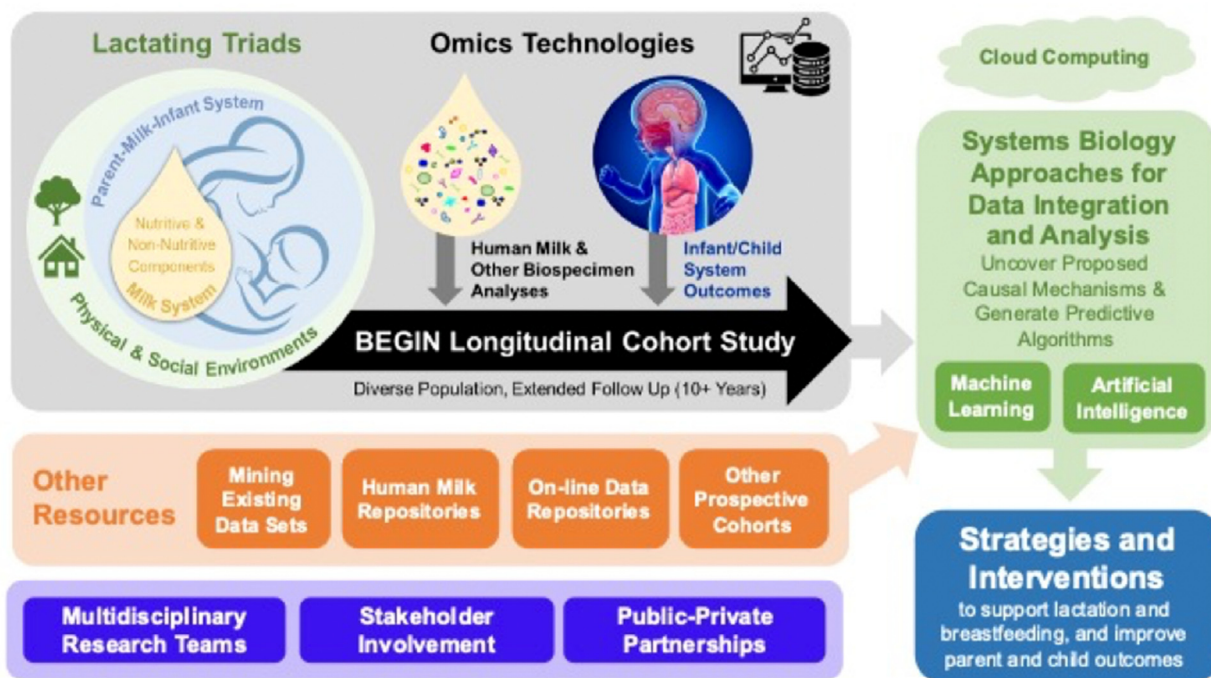


FIGURE 3. Proposed analytical framework to study human milk as a biological system. To further our understanding, multidisciplinary teams of investigators should undertake a longitudinal investigation of diverse cohort of lactating parent–infant dyads. Frequent biological sample collection coupled with extensive paired metadata and multi-omic analyses with enable deep phenotyping of participants. Application of systems biology provides a unique opportunity for in-depth modeling of the role of human milk in the breastfeeding dyad to develop integrated models that predict and explain the role of human milk on a systems level.

Text Box 5

Approaches for gathering existing and future human milk and lactation metadata and biological samples to inform systems biology approaches

1. Establishment of an *online portal* where researchers can upload demographic and biological data from completed and ongoing studies.
2. Creation of a *biorepository* of human milk samples paired with other biological samples, saliva, stool, urine, etc.
3. Development of a core list of metadata to be collected in studies of human milk, lactation, and breastfeeding practices.
4. Utilize validated surveys/questionnaires when possible or validate newly developed surveys/questionnaires
 - a. NIH PhenX Toolkit is a Web-based catalog of recommended protocols [189]
 - b. Human milk researchers should contribute protocols to this site
5. Availability of supplemental funding to support collection of additional metadata and biological sample collection on existing or future single-investigator grants.

framework conceptualized by WG 5 provides an excellent guide to consider how discovery research (T1) could be designed and adapted to inform the other 4 stages: human health implications (T2), clinical and public health implications (T3), implementation (T4), and, ultimately, impact (T5) [4]. The analytical framework to study human milk as a biological system is shown in Figure 3, and the following sections describe specific aspects to be considered in the design of integrated studies of human milk.

Consideration of participant selection

Although some global initiatives are underway [191,193–195], the vast majority of human milk research has been conducted in ethnically homogeneous, relatively Western populations. As discussed in more detail by WG 5, with expansion of the human milk and lactation research enterprise to large comprehensive studies to profile human milk, it will be important to ensure that diversity and representation are considered when recruiting research participants. In addition, due

TABLE 3

Recommendations for data, testing, and biospecimens to study human milk as a system-within-a-system.

Type of Data	External Ecologies	Internal Ecologies	
		Lactating Parent	Infant or Child
Environmental and biological sample collections	<ul style="list-style-type: none"> • Environmental samples • Water, • Soil • House dust • etc. 	<ul style="list-style-type: none"> • Human milk • Blood • Saliva • Oral and nasal swabs • Skin swabs, including nipple and areola • Hair • Feces • Urine • Vaginal swabs 	<ul style="list-style-type: none"> • Blood • Saliva • Oral and nasal swabs • Skin swabs • Hair • Feces • Urine
Physiological measurements or testing or observations		<ul style="list-style-type: none"> • Height • Weight • Body composition • Milk production • Brain structure and functional MRI • Endocrine, immune, and intestinal function (see Table 4) • Biopsychosocial interactions between parent and child, particularly during feeding 	<ul style="list-style-type: none"> • Length or height • Weight • Body composition • Milk intake and mode of feeding • Brain structure and functional MRI • Cognitive function (tested) • Endocrine, immune, and intestinal function (see Table 4) • Biopsychosocial interactions between parent and child, particularly during feeding
Medical records, questionnaires, and surveys	<ul style="list-style-type: none"> • Social determinants of health* • Geography • Religion • Culture • Employment type • Sanitation • Stress • Xenobiotic exposure <ul style="list-style-type: none"> - environmental contaminants, - therapeutic or recreational drugs, - cigarette smoke - other toxins 	<ul style="list-style-type: none"> • Race and ethnicity • Sex • Health history and current status • Medications and supplements • Breastfeeding duration and exclusivity • Sleep patterns • Weight status: prepregnancy, gestational weight gain, recent weight gain or weight loss • Parity • Pregnancy complications • Breast surgery (reduction or augmentation) • Lactation history • Timing of initiation of lactation • Executive function • Physical activity 	<ul style="list-style-type: none"> • Race and ethnicity • Sex • Health history and current status • Medications and supplements • Breastfeeding duration and exclusivity • Sleep patterns • Gestational age • Singleton or multiple birth • Infant formula use and type • Dietary intake (human milk, infant formula, complementary feedings) • Cognitive function (parent reported)

MRI, magnetic resonance imaging.

* Social determinants of health are conditions in the places where people live, learn, work, and play that affect a wide range of health and quality-of-life-risks and outcomes (<https://www.cdc.gov/socialdeterminants/index.htm>).

to the lack of evidence for long-term outcomes of early life nutrition interventions (e.g., from RCTs of specific bioactive components of human milk as discussed previously) it will be critical that the cohort be followed for a minimum of 10 y. Moreover, longitudinal cohorts will need to be adequately powered to account for attrition and participants should provide consent for future analyses. As single nucleotide polymorphisms in the lactating parent or the infant can influence human milk composition or utilization by the infant, studies should apply Mendelian randomization for common single nucleotide polymorphisms when applicable [196,197]. Lactahub, a partnership project of The Global Health Network at the University of Oxford and the Family Larsson-Rosenquist Foundation, recently published a useful framework to guide ethical breastfeeding research and interventions [198].

As we consider conducting research across the globe, decolonizing research is a complex problem that requires a multipronged approach to affect change. Currently a power imbalance exists between HIC and low- or middle-income countries (LMIC), which creates and perpetuates imbalance for field sites. Shifting this imbalance must be a priority in the design of future human milk studies. Specifically greater attention, understanding, and funding need to be allocated for utilities such as electricity to facilitate long-term sample storage at LMIC field sites, clinics, and hospitals, as well as infrastructure to support data capture and development of local bioinformatics cores. Intellectual property must be shared with LMIC collaborators in addition to equal acknowledgment for their contributions. Compensation for researchers and staff in LMIC environs is generally budgeted lower than that of HIC counterparts; this perpetuates the misconception that there is a gap in research capacity. Highly skilled researchers are present in LMIC settings, it is a matter of HIC collaborators recognizing that the capacity exists and budgeting appropriately to retain this expertise. Travel for field site visits and conference presentations is often budgeted for HIC research teams; however, LMIC counterparts rarely receive funding in these areas. Trainee exchange programs must be encouraged, as well as financial support for field site investigators and research teams to present findings in their own right rather than the HIC PI presenting on their behalf.

The value of collaborative team science

The challenge of studying the complexity of human milk and its ecology will require cross-disciplinary teams of investigators representing multiple disciplines (e.g., cultural and physical anthropology, evolution, immune function, mammary gland biology and lactation physiology, medicine, neurobiology, nutrition, and public health) working together in an integrated approach [199]. While scientific research is increasingly conducted by small teams and larger groups rather than individual investigators, the challenges of managing these collaborations can slow these teams' progress in achieving their scientific goals. Thus, best practices for enhancing the effectiveness of research teams should be employed [200].

Biospecimens and data

In addition to the collection of human milk itself (see [201] for a detailed guide to human milk collection and storage), the study of human milk as a "system-within-a-system" will require additional biological specimens from the lactating parent and infant. Depending on the research question, it may be important to collect blood, urine, infant saliva (a source of human milk microbes and other 'communication signals'), serum (to monitor nutritional status or hormones), skin, stool, and nasal swabs (to analyze the gut, dermal, and airway

microbiomes, respectively). Blood, saliva, urine, feces, and vaginal and skin swabs from the lactating parent may also be relevant.

To assess the impact of lactating parent [1] and infant [3] inputs on human milk composition, it will be necessary to capture information on such factors as dietary intake, feeding practices (frequency, pumping, complementary feeding, etc.), lactating parent and infant health conditions, sociodemographics, as well as the physical and social environment. Tables 3 and 4 summarize the recommendations for sample and data collection and analyses, respectively, to support systems biology analyses. Although logistically challenging, capturing milk volume (produced by the lactating parent and/or consumed by the infant) provides extremely useful information and would be necessary to determine lactating parent inputs and infant exposure. Researchers are also encouraged to use relatively noninvasive approaches for repeated measures of cognitive development (e.g., magnetic resonance imaging [MRI]) [202,203], body composition (air displacement plethysmography, quantitative nuclear magnetic resonance or dual X-ray absorptiometry) [204–206], or intestinal gene expression using exfoliated epithelial cells [35].

Timing and procedure of sample collection

As described by WG 1-3 [1–3], a unique chronobiology exists for human milk composition involving both parental and infant inputs. Temporal changes in human milk composition occur over the course of a feeding, a day and throughout the period of lactation. Timing is thus an important consideration when designing human milk studies.

TABLE 4

Proposed sample analyses to study human milk as a biological system and interactions within the lactating triad.

Sample Type	Human Milk (Colostrum, Transitional and Mature)	Lactating Parent	Infant or Child
Genome: Single nucleotide polymorphisms	Cells from lactating parent	Blood Saliva	Blood or saliva
Epigenome: DNA methylation	Cells from lactating parent	Blood Oral or nasal swabs	Blood Oral or nasal swabs Saliva
miRNA/exosomes	Human milk	Saliva Blood	Blood
Nutrients: Proteins, lipids, HMO, vitamins, minerals, trace elements	Human milk	Blood Urine	Blood Urine
HMO	Human milk	Blood Feces Urine	Blood Feces Urine
Microbiome and metagenome: Bacteria, fungi, Archaea, viruses	Human milk	Feces Saliva Swabs: Oral, nasal, vaginal, skin (including nipple and areola)	Feces Saliva Swabs: Oral, nasal, skin
Metabolome and proteome	Human milk	Blood Feces Urine	Blood Feces Urine
Lipidome	Human milk	Blood	Blood
Immune cells and proteins	Human milk	Blood	Blood
Hormones and growth factors	Human milk	Blood	Blood

HMO, human milk oligosaccharide.

Ideally, the time of day and time postpartum should be standardized across participants, or at least clearly documented so that these parameters can be accounted for during data analysis. Depending on the research question, it may be especially relevant to collect colostrum, transitional, and/or mature milk. Longitudinal sampling can offer important insights about compositional changes over time and can link human milk composition to short- and long-term health outcomes.

In addition, how the human milk sample was collected is a crucial design component as numerous factors can influence accuracy of milk composition. These factors are described in detail elsewhere [201,207,208]. Although many methodological factors (e.g., human milk sampling, handling, and analytics) are known to impact human milk composition, few studies have investigated those as primary outcomes or variables, making it an important area of future research in human milk [207]. Examples of factors to consider are shown in **Text Box 6**.

Human milk analysis

In addition to the coverage of human milk components offered by WG 2, readers are referred to the recent book summarizing milk sampling and nutrient and macromolecule analysis [209]. It is important to consider at the study design phase which analytes will be analyzed (or might be in the future), as this will inform sample collection, analysis, storage, and requisite resources needed. For example, some components are highly sensitive to light and/or freeze-thaw cycles, whereas others are unaffected [201,207]. It is also important to ensure that assays have been validated for human milk. As for any large study, appropriate controls and standards should be included to address and adjust for potential batch effects.

Integration: computational systems biology

The previous sections illustrate the myriad of internal and external ecologies that may affect human milk composition and its impact on the lactating parent–infant dyad. Due to this complexity, outcome-based studies on a population level often only cover very specific aspects of human milk ecology. At the same time, recent advances in high-throughput technologies have provided access to multidomain and multi-omic data, enabling unprecedented insights into complex biological systems from multiple viewpoints such as the microbiome, proteome or metabolome, as well as a wide range of other omics [210]. Such technologies will require systems biology approaches to study human milk as a complex biological system [6], unraveling the underlying processes that influence human milk composition, and its effects on infant development and clinical/functional outcomes in both infant and lactating parent.

In contrast to population level approaches, multi-omic studies typically exhibit different data characteristics and challenges (**Text Box 7**). High-throughput multi-omics data poses unique challenges and requires sophisticated computational tools to analyze. For this, a wide variety of methods for multi-omics integration are currently being developed at the intersection of bioinformatics and machine learning [211–214]. This includes methods based on specific fields like Bayesian modeling [215,216], network analysis [213,214] as well as deep learning [217,218], and considerations on sample size requirements and estimation [219,220]. In addition, there are community-driven efforts to maintain an overview of relevant work and software packages [221].

While integrating multiple omic datasets is challenging, it has been demonstrated to improve the capacities of models for biological processes in the context of the lactating parent–infant dyad, particularly as they relate to pregnancy outcomes. For example, several studies illustrate that combining the information from a wide variety of omics—including transcriptome, or proteome, and more—can be used to model gestational age [222], as well as improve the performance of predictive models for adverse pregnancy outcomes like preterm birth [223], preeclampsia [224], or gestational diabetes [225]. Additionally, these models help to elucidate complex biological systems, as exemplified by a recent study mapping the underlying processes of onset of labor [226].

Applying and adjusting these methods to studying human milk holds great potential to uncover the complex processes involved in the triad from a systems biology viewpoint. A particular challenge in this context is to integrate population level studies with multi-omics approaches due to the demanding collection process of synchronized omic measurements as well as the technical aspects of integrating omics with nonomics records, such as clinical meta data [227,228]. However, multimodal models have been previously applied in biological data integration [229] and are particularly relevant for multi-omics analysis and integration [147,214] with the potential to combine modalities across regular tabular data, time series, images, as well as text into a joint holistic model for a multitude of predictive settings [230,231].

Human milk and feeding within the breastfeeding dyad influence infant development as well as various clinical/functional outcomes. These effects are highly interrelated and may point toward intertwined underlying processes. This interrelatedness can be studied and can be exploited by using novel concepts from machine learning in the field of multitask learning [232]. Multitask learning takes advantage of information contained in related outcomes to make models more robust by preferring solutions that share common (e.g., biological) structures across these outcomes [233]. Here, deep learning has recently contributed significantly to advancing the field of multitask learning [234,235].

In summary, recent advances in machine learning, including multimodal learning, multi-view representation learning [230,236] and multitask learning [233,235] provide a unique opportunity for in-depth modeling of the role of human milk in the breastfeeding dyad. These areas of research aim to combine datasets from various modalities and across different tasks (e.g., prediction of outcomes) to develop integrated models that predict and explain the role of human milk on a systems level and can apply techniques currently used in other areas, such as microbial community ecology [237]. The combination of multimodal learning with multitask approaches [238] allows for condensed representations of the inputs and the modeled phenotypes (outcomes), which may lead to a novel holistic understanding of the underlying processes associated with human milk and its influence on infant development as well as clinical/functional outcomes.

Text Box 6

Factors to consider for standardizing human milk sampling

- Time of day and if coincident with routine feeding times?
- Time postpartum.
- If or how the breast was cleaned prior to sample collection.
- Was the milk collected by manual expression or breast pump? If a pump, what type?
- Does the milk sample represent an aliquot of a full breast expression or milk sampled early, mid or late during a feeding?
- Were both breasts sampled or, if only one, left or right?
- How was the sample stored after expression, for how long and in what type of container (bag, plastic bottle, glass bottle, etc.), was a preservative added? [207]

Text Box 7

Analytic challenges of multi-omic studies

- Large numbers of measurements across several omics technologies per sample, while the number of samples is small (commonly machine learning models are trained and optimized on datasets with many more samples than measurements)
- Different omics techniques are highly heterogeneous in individual characteristics and number of features (e.g., sparse microbiome data vs. targeted proteomics assays). In particular, sparse data with many undefined values require different processing and model imperatives than data with dense input, and
- Omics data of high dimensionality, but with low information content, may preclude information from smaller, more dense omics to be included in a model. Stack generalization has previously been utilized successfully to address this challenge
- Omics data from various study sites may be impacted by a combination of genetic, environmental, and technical factors. Careful use of machine learning algorithms can enable the development of generalizable models despite these variations

Case study 1—interactions of human milk components and cow milk allergy

Recently, hierarchical cluster analyses have been used to assess correlations between immune factors in human milk showing that those factors (IL-6, IL1 β , IL-10, thymic stromal lymphopoietin, TGF- β 1) that were correlated with infant cow's milk allergy as an outcome were actually independent of maternal atopy [58]. A follow-up study in a farming lifestyle cohort utilized systems level network analysis to identify communities of multiple human milk factors [239]. Path-based analysis of HMO showed lower activity in the path involving lacto-neohexaose in the farming lifestyle mothers as well as higher levels of LNnT and 2 long-chain fatty acids (stearic acid and tricosanoic acid) compared with urban lifestyle mothers. Human milk from both groups formed 5 different clusters, e.g., butyrate production was associated with *Prevotellaceae*, *Veillonellaceae*, and *Micrococcaceae* cluster. Development of atopic disease in early childhood was more common in urban lifestyle and associated with lower levels of total IgA and IgA₂ to dust mite, as well as of thymic stromal lymphopoietin. Thus, external ecologies (traditional, agrarian lifestyle, and antibiotic use) were shown to be strong regulators of immune and metabolic factors derived from the lactating parent, which may have downstream implications for postnatal developmental programming of infant's gut microbiome and immune system [239].

Case study 2—lactating parent–human milk–infant interacting microbiomes

In the BEGIN Project Executive Summary [5], lactating parent–human milk–infant interacting microbiomes were highlighted as a Case Study for the need to apply an ecological systems biology approach. A consortium of microbes, viruses, archaea and fungi and protozoa are present in human milk and are proposed to play a role in early infant colonization [240]. The large number of host, microbial, and medical factors that can influence the human milk microbiota complicate our understanding of the factors driving heterogeneity in the human milk microbiome [195]. Furthermore, the relationships between the human milk microbiome and infant microbial colonization are poorly understood, with 2 studies reporting that 25% [241] or >70% [242] of the infant fecal genera originate from human milk; however, neither study reported strain level analysis. Expression of human milk via pumping also affects the human milk microbiome [243] and is often not reported in studies of the human milk microbiome.

In the CHILD cohort, co-occurrence of specific bacteria in human milk and infant feces (including *Streptococcus* spp. and *Veillonella dispar*) were observed within mother–infant dyads, which was reduced when infants received pumped human milk in a bottle [244]. The commonly shared human milk bacteria were associated with the 3-month-old infant gut microbiota, explaining 1.0% of the variation

(R^2_{adj}). While this is a low percentage, it was greater than that explained by breastfeeding exclusivity (0.76%), birth mode (0.87%), intrapartum antibiotics (0.72%), or older siblings (0.58%) [244]. Multivariate analyses were applied to investigate the contribution of bacterial communities across the lactating parent–infant dyad (maternal and infant saliva, human milk, maternal and infant feces) on human milk and infant fecal bacterial composition [85]. As might be expected, the human milk microbiome was more similar to the infant oral microbiome than the infant fecal microbiome. Canonical correlation analysis suggested strong associations between the human milk genera and all other sample types, although sources of >87% of the infant fecal genera were unknown based on SourceTracker2. However, this might be a limitation of the program and the depth of sequencing because species or strain level resolution was not reported [85]. Furthermore, only 52% of infants were exclusively BF at 3 mo of age and use of breast pumps was not reported [85]. Another study also utilizing SourceTracker identified infant gut microbiome composition more similar to sibling stool samples than human milk [245]. Taken together, these data demonstrate potential relationships between human milk and infant fecal microbiota; however, more comprehensive data collection and human milk sample analyses are required to delineate other ecological factors within the lactating parent–human milk–infant triad that are accounting for infant microbiome composition.

Overall Conclusions

While there are notable challenges in the study of human milk as a complex biological system, emerging scientific and analytical advances are uncovering unprecedented opportunities to study the biology of human milk and uncover the internal and external ecologies that drive outcomes observed in the breastfeeding triad over time and across a myriad of conditions. Considerations for the design of human studies of these complex relationships, including the integration of new approaches in multi-omic data collection, computational systems biology and approaches to the use of large complex datasets, will be important to advance the field of human milk and lactation research. Unprecedented opportunities await the field to uncover new mechanistic interactions between the triad of the human milk biological system. The challenges remain great, but the return will be greater.

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Author contribution

The contributions of the authors were as follows—DJR conceptualized the project; all authors contributed to writing the manuscript: Introduction led by DJR and SMD, Section 1 led by AA, SEC, SMD, KMJ, WL, and CMS; Section 2 led by SEC, SMD, BL, and CMS; Section 3 led by NA, MBA, MB and SMD. SMD, DJR and AS prepared the final draft of the manuscript, which was read and approved by all authors.

Conflicts of interest

All authors have declared no conflicts of interest related to existing financial arrangements with any company or organization sponsoring this manuscript. (AS is an employee of the Academy of Nutrition and Dietetics).

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