Report of a Meeting

Assessing the safety of bioactive ingredients in infant formula that affect the immune system: recommendations from an expert panel

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ABSTRACT

Bioactive ingredients for infant formula have been sought to reduce disparities in health outcomes between breastfed and formula-fed infants. Traditional food safety methodologies have limited ability to assess some bioactive ingredients. It is difficult to assess the effects of nutrition on the infant immune system because of coincident developmental adaptations to birth, establishment of the microbiome and introduction to solid foods, and perinatal environmental factors. An expert panel was convened to review information on immune system development published since the 2004 Institute of Medicine report on evaluating the safety of new infant formula ingredients and to recommend measurements that demonstrate the safety of bioactive ingredients intended for that use. Panel members participated in a 2-d virtual symposium in November 2020 and in follow-up discussions throughout early 2021. Key topics included identification of immune system endpoints from nutritional intervention studies, effects of human milk feeding and human milk substances on infant health outcomes, ontologic development of the infant immune system, and microbial influences on tolerance. The panel explored how "nonnormal" conditions such as preterm birth, allergy, and genetic disorders could help define developmental immune markers for healthy term infants. With consideration of breastfed infants as a reference, ensuring proper control groups, and attention to numerous potential confounders, the panel recommended a set of standard clinical endpoints including growth, response to vaccination, infection and other adverse effects related to inflammation, and allergy and atopic diseases. It compiled a set of candidate markers to characterize stereotypical patterns of immune system development during infancy, but absence of reference ranges, variability in methods and populations, and unreliability of individual markers to predict disease prevented the panel from including many markers as safety endpoints. The panel's findings and recommendations are applicable for industry, regulatory, and academic settings, and will inform safety assessments for immunomodulatory ingredients in foods besides infant formula. *Am J Clin Nutr* 2021;00:1–18.

Keywords: bioactives, development, human milk, immune system, immunomodulation, infant, infant formula, nutrition, safety recommendations

Introduction

The importance of nutrition is heightened during infancy, a period of rapid growth and development. Because many organ systems continue to develop postbirth, optimal nutrition during this period sets the stage for a healthy start to life. Moreover, growing evidence suggests that certain dietary components influence immune system development, and that the first year of life is a period during which major developmental immune

Portions of the panel's work were presented at a satellite symposium at the American Society for Nutrition annual meeting in June 2021 in order to receive inputs from a wide group of interested parties to help inform the panel's final report.

Unrestricted educational grant to the Columbia University Institute of Human Nutrition from the Infant Nutrition Council of America (INCA). The expert panel cochairs were responsible for the design, implementation, analysis, and interpretation of the panel's work; neither INCA nor its members were involved in any of those capacities. INCA and its members were invited to ask questions of panel members following their presentations at a November 2020 symposium related to the topic of this report.

Supplemental Tables 1–2, Supplemental Material, and Supplemental Figures 1–3 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: CFSAN, Center for Food Safety and Applied Nutrition; GRAS, generally recognized as safe; IOM, Institute of Medicine; ILSI, International Life Sciences Institute; RCT, randomized controlled trial.

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milestones occur and form the foundations of lifelong immune homeostasis.

Human milk contains bioactive substances and immune active factors that support infant health, growth, and development (1). Breastfeeding is the gold standard for optimal nutrition during infancy (2, 3), but the high prevalence of infant formula feeding in the USA (4) and elsewhere has contributed to increasing academic and commercial interests in adding novel bioactive ingredients to infant formula to better simulate the composition of human milk and to reduce disparities in health outcomes between breastfed and formula-fed infants.

Because many bioactive ingredients are not classical "toxicants," the usefulness of traditional food safety assessment methodologies may have limitations for understanding the potential effects of their use in infant formula. As a result, a need has emerged to develop an appropriate framework to evaluate the safety of novel bioactive ingredients for such use.

In 2020, the Institute of Human Nutrition at Columbia University convened an 11-member expert panel representing expertise in pediatrics, nutrition, immunology (including basic, clinical, and translational research in these disciplines), and regulation of foods for special dietary uses (see **Supplemental Table 1** for a listing of panel member names, affiliations, and expertise). Panel member selection criteria included demonstrated expertise and the 2 panel cochairs aspired to attain balanced representation between nutrition and immunology, gender and age balance, and to weight the panel with US scientists but also include perspectives from outside the USA. (See panel member disclosures in the Acknowledgments section.) The panel's charge was to recommend a comprehensive yet practical set of clinical assessments and immune system measurements that demonstrate reasonable certainty of no harm, for healthy term infants, for novel bioactive ingredients intended for addition to infant formulas. The panel was also charged to consider both clinical and immune system assessments that could indicate efficacy in healthy term infants, but this objective was secondary to developing recommended safety assessments. The panel limited its scope to the use of infant formula as the vehicle for feeding bioactive ingredients to infants.

The US Regulatory Context for Evaluating the Safety of Infant Formula

The process of determining the safety of new ingredients in infant formula has evolved over the past 2 decades. In the regulatory context of the US food system, "safe" or "safety" means that reasonable certainty exists in the minds of competent scientists that the substance is not harmful under conditions of its intended use (5). Ascertaining whether a food substance is harmful requires experts to assess its effects on the human body and evaluate whether any changes that occur are biologically or clinically meaningful.

The US FDA outlines recommended toxicological testing to evaluate the safety of new substances proposed for use in food (6). A challenge to applying general toxicology approaches, which typically involve doses well above the intended use concentration to determine concentrations at which no effects and no adverse effects are observed, to nutrients is that feeding at multiple concentrations of intended exposure is not always possible.

There are special concerns regarding infants, a vulnerable population, because novel bioactive preparations presented early in life might perturb normal immune system development. Furthermore, modes of action and response curves associated with the anticipated effects of bioactive ingredients proposed for use in infant formula are often not known nor elucidated and may be nonlinear; it is possible that they could have subtle, but long-term effects on infant development (7).

The only ingredients that may be used in infant formula in the USA are those safe and suitable for such use as shown by food additive approval, prior sanction, or GRAS (generally recognized as safe) determination (8). The **Supplemental Material** includes further information about GRAS and FDA guidance on toxicological testing to evaluate the safety of new substances proposed for use in food, and specific requirements for infant formula.

Challenges associated with analyzing the immune system

A number of challenges exist for both manufacturer demonstration and regulatory assessment of the safety for novel bioactive ingredients in infant formula. First, immunological research methodologies and markers are quickly advancing and broadening both current understanding and unresolved questions on the ontogeny of immune development. Second, the term "immunomodulation" is not precisely defined, yet is increasingly used to describe nutritional interventions with potential to modify the immune system. Examples of immunomodulation are described in the FDA Center for Food Safety and Applied Nutrition's (CFSAN) electronic reading room, which publicizes selected records of CFSAN correspondence on bioactive ingredients for infant formula use (9). These include alterations in immune cell populations or states of activation and responses of immune cells to various stimuli and balance of activities between Th1/Th2 arms of the developing adaptive immune system. Third, safety issues related to immunomodulation do not necessarily create a detectable pathological state during infancy and cannot be reliably addressed with standard toxicological approaches or other clinically accepted methodologies. The question then becomes whether alterations in immune cell populations or states of activation present a safety concern, and what modifications are in the range of normal versus disease. For concerns about long-term effects, it is difficult to determine whether any outcomes that develop were influenced by variables introduced in infancy, and if so, what effect did nutritional factors have relative to other potentially contributing factors.

Because of these challenges, a regulatory need has emerged to define a process of evaluating the safety of bioactive ingredients proposed for use in infant formula. Two prior efforts are particularly relevant to this topic. The first, a 2004 Institute of Medicine (IOM) report, issued recommendations for evaluating the safety of new ingredients in infant formula, including consideration of immune system effects (10). The second, a 2005 report from an expert group convened by the International Life Sciences Institute (ILSI) Europe, proposed markers with high suitability for assessing immune function in healthy human subjects, based on biological relevance (known correlation with clinically relevant endpoints), sensitivity (within- and between-subject variation), and feasibility (11). Substantial advancements in immunology and ontogeny of infant immunity have occurred

since publication of these reports, alongside a proliferation of the development of new, bioactive, potentially immunomodulatory ingredients for use in infant formula. Given this context, the expert panel was convened to recommend endpoints for assessing the safety of novel bioactive ingredients in infant formula, with a focus on the immune system.

Expert Panel Methods and Approach

The panel reviewed background information, including correspondence on GRAS notifications for novel bioactive ingredients in infant formula as well as scientific literature describing the trajectory of infant immune system development and the potential effect of nutritional interventions. In November 2020, panel members participated in a 2-d virtual symposium during which they delivered scientific presentations and discussed preselected questions on topics relating to the timepoints for potential safety assessments, the usefulness of particular analytes in safety assessment, appropriate comparators, and research gaps. Following the convening, panel members participated in additional meetings as a full panel and/or in subgroups to review specific types of clinical and immune system assessments to consider. All panel members reviewed and agreed to the panel's final recommendations and supporting rationale. A draft manuscript was reviewed by selected stakeholders in academia and industry, and revised in response to their feedback, before submission for publication.

The panel regularly referenced a number of key terms, defined in **Box 1**, during the process of developing its recommendations.

Box 1:

Key Terms Used in this Article

Bioactive: A term for which a widely adopted standard definition does not exist, used in this article to refer to an ingredient that has a physiologic effect on the human body.

Dysbiosis: An unhealthy state of altered and inappropriate microbial colonization of mucosal and epithelial surfaces, increasingly recognized as a potential contributor to adverse health outcomes.

Efficacy: An outcome resulting from the addition of a new ingredient to infant formula whereby the ingredient confers a health and/or developmental benefit to the infant, compared to the same infant formula without the new ingredient.

Immunomodulation: Any significant change in an outcome or immune system measurement.

Ingredient: Nutrients (defined below) or other bioactive substances added to infant formula.

Nutrient: Any vitamin, mineral, or other component required in infant formula, per section 412(i)(1) of the Federal Food, Drug, and Cosmetic Act or by regulations issued under section 412(i)(2) or that is identified as essential for infants by the Food and Nutrition Board of the National Academy of Medicine through its development of a DRI, or that has been identified as essential for infants by the FDA through a Federal Register publication (12).

Safety (statutory definition): Reasonable certainty that a substance is not harmful under the conditions of its intended use, as determined by common knowledge throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food (5).

Science to Inform the Recommendations

During the November 2020 convening, each panel member delivered a presentation designed to inform the panel's deliberations about assessing the safety of bioactive ingredients in infant formula. **Box 2** highlights the points from each panel member's presentation that were most salient to informing the panel's key considerations (**Box 3**) and the guiding criteria (**Box 4**) that led to its recommended endpoints for assessing the safety of novel bioactive ingredients in infant formula. The Supplemental Material contains the full presentation summaries and associated citations.

Box 2:

Key Points from Expert Panel Member Presentations at the November 2020 Convening

Ronald E Kleinman: The US regulatory process to evaluate the safety of new food ingredients, with emphasis on the GRAS system

- New ingredients in infant formula are typically determined to be safe and suitable for such use via the GRAS notification process.
- Petitions to support the safety of an ingredient must fully characterize the ingredient and specify concentrations in which it is to be added to specific categories of foods to produce specific technical or functional effects.
- In terms of potential immune markers for assessing the safety of novel food ingredients, a robust starting point may be the safety assessments used in GRAS determinations for α-lactalbumin and *Bifidobacterium animalis* subsp. *lactis* AD011, the markers suggested by the ILSI Europe expert group, and the assessments recommended in the 2004 IOM report.
- Safety determination ensures that adverse health outcomes do not occur at higher rates or with increased severity among infants consuming infant formula containing the novel ingredient.

Frank Greer: Ingredients (already) added to infant formula with potential to modify immune status

- Little evidence exists to demonstrate that ingredients required in infant formula under the Infant Formula Act and its amendments—vitamins A and C, B-vitamins (including folic acid), vitamin D, vitamin E, iron, zinc, or selenium, at greater than the required amounts—meaningfully alter immunological responses in healthy, well-nourished infants.
- Ingredients that provide nutrients not required by FDA regulations have been included or proposed for addition

in (term) infant formula (largely via the GRAS process), and immune system endpoints have been examined in relation to modifying infant formula with some of these ingredients.
- Research interest in such ingredients tapers off after their inclusion in infant formula, and mechanisms of action to demonstrate any immune system effects of these ingredients often remain to be determined.

Olle Hernell: Endpoints reported in clinical studies in infants for the milk protein ingredients bile salt-stimulated lipase, osteopontin, and milk fat globule membrane fractions

- Bile salt-stimulated lipase, osteopontin, and milk fat globule membranes are 3 examples of bioactive ingredients that have been examined clinically for potential addition to infant formula.
- Although additional work is needed to further clarify the potential effects (including immune system effects) of these 3 ingredients on infant health outcomes, the safety outcomes examined in human studies of these ingredients—such as growth velocity, neurocognitive development, gastrointestinal intolerability, oral and fecal microbiome and plasma and fecal metabolomics, necrotizing enterocolitis, infections, fever, cytokine profile, and vaccine response—provide examples of outcomes to consider in the safety assessment of novel bioactives in infant formula.

Catherine J Field: The effect of nutrients on vaccine responses during infancy

- Vaccine response is a potential proxy measure to predict the effect of modifying infant formula ingredients on resistance to infection or on incidence of infectious immune system-related diseases.
- Vaccine response has been assessed for some ingredient additions to infant formula, contributing to the assessment of safety.
- Because vaccines vary by timing of administration, dosing, and mode of action, differences in immune system responses and timing of those responses are expected for different types of vaccines.
- A combination of different assays and measurements at repeated intervals postvaccination provides the opportunity to collect more detailed information to compare treatments.
- Collaboration with vaccinologists, carefully controlled vaccine administration, and determination of minimum requirements for collection of relevant information are key if vaccine-related endpoints are used to examine the safety and/or efficacy of adding bioactive ingredients to infant formula.

Kinga K Smolen: Ontologic development of the immune system during the first 6 mo of life

- The infant immune system has been described as "differentially adapted" rather than immature or less developed (compared with an adult's); gaps remain in current understanding of specific differences and their biological reasons.
- Normal immunity encompasses significant inter- and intraindividual variation and is influenced by a wide range of intrinsic and extrinsic factors. The latter include mode of birth delivery, mode of feeding, exposure to antibiotics, geography, maternal health and diet, and household pet exposure.
- Many changes occur in the infant immune system during the first year of life. Age-specific resolution of the ontogeny of immunity is not yet fully characterized, but early immune system development appears to follow a stereotypic pattern in preterm and term infants.
- Factors that influence immune ontogeny also influence the effect of nutritional exposures on immune outcomes.
- The emerging field of systems biology can help to address knowledge gaps in infant immune development.

Josef Neu: How studies on preterm infants help define normal immune system development

- The innate intestinal immune system is comprised of both physical and chemical barriers to pathogens, which are affected by environmental factors—such as dietary bioactives—that influence the microbiome, metabolome, and host.
- According to a longitudinal systems-level analysis, immune systems of preterm ($<$30 weeks of gestation) and term ($\geq$37 weeks of gestation) infants differ at birth but converge onto a shared trajectory of development (despite vastly different early-life environmental conditions between the 2 groups), seemingly driven by microbial interactions and hampered by early gut bacterial dysbiosis.
- Dysbiosis is increasingly recognized as a potential contributor to adverse outcomes that sometimes occur in preterm infants, such as necrotizing enterocolitis.

Kirsi M Järvinen: Using allergy to define abnormal, development of IgE, humoral immunity, and tolerance using human milk modulators as a guide

- Observational studies on breastfed infants, including studies that compared outcomes when human milk contained high versus low concentrations of bioactive substances, have helped to identify immune system outcomes to be examined in infant formula studies.
- Human milk substances that could affect immune development and outcomes (such as human milk oligosaccharides, IgA, and cytokines) are influenced by maternal and environmental factors. The interplay of factors makes it difficult to isolate any effects of specific human milk substances on outcomes of interest (e.g., allergic diseases), which would be valuable when examining the effects of these substances added to infant formula. However, evidence on these relations is scarce and reference concentrations for human milk substances do not exist.

Joshua Milner: Abnormal clinical and serological immune system markers in disease states; how disease states help define normal

- When considering supplementation of infant formula with a bioactive ingredient, it is important to consider together the different metabolic pathways that might be activated in different cell types and how such activation could manifest clinically in healthy infants and in infants with common genetic disorders.
- Both rare and common genetic heterogeneity may lead to heterogeneous responses to environmental exposures, including diet, during immune development.
- Nutrient or other bioactive ingredient supplementation may differentially modulate regulatory and effector functions because these substances are metabolized differently by regulatory cells and effector cells.

Talal Chatila: Immune tolerance development in the gut: Regulatory T cells and the microbiota

- Immune system development progresses through distinct stages from birth and is influenced by "seeding" of maternal microbiota (e.g., through the vaginal canal, physical contact, and human milk) that shape the infant's endogenous microbiota and immune responses (**Supplemental Figure 1**).
- The introduction of solid foods triggers a reaction whereby gut immune cells are primed for tolerance such that they develop memory of antigens presented to them in the gut and can mount a healthy response to future exposure to those antigens.
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Key Considerations to Inform Development of Recommended Endpoints

The expert panel discussed several key considerations in the process of developing its recommendations (**Box 3**).

Box 3:

Key Considerations for Developing Recommended Endpoints to Assess the Safety of Bioactive Ingredients in Infant Formula

- Considering the different types of data needed to establish safety and to demonstrate physiologic effects (efficacy).
- Determining the age of interest for measurements.
- Using data on human milk composition and health outcomes of breastfed infants.
- Addressing complexities associated with many bioactive ingredients of interest.
- Identifying suitable markers of developmental immune competence.
- Developing comprehensive and pragmatic recommendations.

Considering the different types of data needed to establish safety and to demonstrate physiologic effects (efficacy)

As outlined in the Introduction, the panel's primary charge was to recommend a comprehensive yet practical set of clinical assessments and immune system measurements that indicate general recognition of the safety of new bioactive ingredients intended for use in infant formulas. Because this charge is directly related to a regulatory objective, i.e., to facilitate a decision about whether a new ingredient poses reasonable certainty of no harm and can be added to infant formula in the USA, the panel determined that safety was its primary focus.

The panel acknowledged that demonstrating efficacy of new bioactive ingredients is of keen interest to stakeholders in industry, research, and regulatory settings (among others), and that evidence of efficacy (i.e., an improved outcome compared with a control group) can also fall under the category of safety (i.e., equal/null effect on outcomes or improved outcomes compared with a control group). It also recognized that with regard to health claims in the European Union, new substances proposed for use in food must demonstrate both safety and efficacy (13). Although the panel was not charged to offer its opinion on the terms of the US regulatory standard, it determined that efficacy outcomes are generally more long term and exploratory in nature and thus of secondary importance to its charge, but wholeheartedly agreed to encourage research that examines efficacy endpoints. Such research would ideally include well-designed randomized controlled trials (RCTs) to examine designated endpoints in specific populations. Examples of relevant research gaps and priorities are included in the section titled Research Gaps.

Determining the age of interest for measurements

The panel recognized that the effects of early life dietary intervention may manifest over both the short and long terms, and that longitudinal studies designed to examine the latter type of outcomes are relatively scarce for bioactive ingredients of interest. It also examined evidence on development of the infant immune system, including the role of gut microbiota. The panel's findings led it to determine that the first 6 mo of life is an appropriate time period during which to conduct dietary interventions of bioactive ingredients with potential to modify the infant immune response. It also determined that an appropriate observation period during which to conduct the safety assessments outlined in **Tables 1** and **2** is the first 12 mo of life.

Time period for intervention: First 6 mo of life.

Rapid immune system cell expansion and functional maturation occurs during the first 6 mo of life, bracketed by major stimuli to the immune system of birth and introduction of weaning foods (14). Activities that occur during this time include imprinting of cells in the immune system and development of immunological memory and tolerance (15). In addition, several key immune cell populations (dendritic cells, B cells, natural killer cells) reportedly reach adult-like phenotypes during the first 3 mo of life (16). Although much remains to be learned about the age-dependent maturation of immune system

TABLE 1 Recommended standard clinical endpoints and laboratory measures to demonstrate safety for immune-related outcomes in infants from birth to the age of 6 mo

Endpoint	Associated symptoms	Reference ranges or method of diagnosis
Appropriate growth and growth velocity		
Length, weight, head circumference		WHO growth curves for 0–12 mo
Infection (difference in incidence of infections and associated symptoms; time to recover from infections)		
Gastroenteritis	Fever, diarrhea, blood and/or mucous in stool, vomiting	– History and physical exam – Appropriate confirmatory laboratory studies where indicated (e.g., stool cultures, BMP, CBC, ESR)
Ig isotypes: IgG, IgM, IgA		Age-based reference values (available for cord blood and for infants at age 1 mo, 6 wk, and 2, 3, 4, 5, 6, 7–9, and 10–12 mo) (61)
Otitis media	Fever, pain	– Physical exam – Consider confirmatory otoscopy
Pneumonia (or viral-induced bronchiolitis)	Fever, respiratory distress, cough	– Physical exam – Appropriate confirmatory laboratory studies (CBC, chest X-ray, and other studies as indicated)
Urinary tract infections	Fever	History and physical exam and appropriate confirmatory laboratory studies where indicated (e.g., CBC, urinalysis, urine culture)
Other adverse effects related to inflammation	Persistent/intermittent vomiting, reflux, food refusal, irritability, diarrhea, abdominal pain, and associated growth failure/failure to thrive	Confirmatory laboratory studies: CRP, fecal calprotectin (reference standards for age), serum albumin, tissue biopsy
Response to 1 or more vaccines administered during the first 6 mo of life (62)[1] (Ideal to be done at >1 time point after the vaccine and to include a baseline/prevaccine measure. Timing of measurement depends on the vaccine and when it is administered)		– Confirm no acute safety issues (63) – Evidence of successful seroconversion in plasma, serum, saliva, or stool, determined by vaccine-specific antibodies ($IgG_{isoforms}$, IgA, or IgM, depending on type of vaccine used), and using ≥2 different types of vaccines (64) – Assays to measure neutralization of pathogens may also be considered
Variables related to infections		
Antibiotic use		– Prescription of antibiotics to treat infection(s) – Review of medical records or by physician report
Hospitalizations		Admission to hospital due to infection(s)
Allergy and atopic diseases		
Allergic rhinitis (hay fever)	Sneezing, nasal congestion, rhinorrhea	– History and physical exam – Confirmatory allergen testing where appropriate
Atopic dermatitis	Erythema, edema, crusts, excoriations, lichenification, dryness, degree of itch, loss of sleep	– History and physical exam – Confirmatory allergen testing where appropriate
Food allergy (IgE mediated)[2]	Itching/swelling of lips/mouth/throat, urticaria, severe vomiting or diarrhea, respiratory signs, or anaphylaxis within 2 h of ingestion of a specific food	Confirmatory laboratory studies: skin prick tests; specific IgE antibody testing (RAST); IgE concentrations; specific food elimination followed by oral challenge tests
Eosinophilic gastrointestinal disorders (EGIDs)[3]	Reflux, nausea, vomiting, food refusal, dysphagia, abdominal pain, feeding issues, irritability, weight loss, failure to thrive, food impaction	High eosinophil counts in esophageal and/or intestinal biopsies
Allergic proctocolitis	Blood in stools	Assess stool for visible blood, remove suspected food allergens in diet (including mother's diet if breastfeeding) and assess for resolution of symptoms
Food protein-induced enterocolitis (FPIES)	Delayed episodes of vomiting and lethargy 2–4 h postingestion, followed by diarrhea; blood in stool	– History and physical exam – Eliminate suspected ingredient, followed by feeding test; if problem remains consider endoscopy to rule out other causes

(*Continued*)

TABLE 1 (*Continued*)

Endpoint	Associated symptoms	Reference ranges or method of diagnosis
Recurrent wheezing	Wheezing	– History and physical exam – Chest X-ray where indicated – Physician or care provider reports

[1]Comparison of vaccine response in formula-fed infants to reference breastfed infants should recognize factors that may affect the response, e.g., geography, season, iron status, sex, maternal vaccinations (40, 65–67). BMP, basic metabolic panel; CBC, complete blood count; CRP, c-reactive protein; ESR, erythrocyte sedimentation rate; RAST, radioallergosorbent test.

[2]Exclusively breastfed infants typically do not develop IgE-mediated food allergic reactions in response to food allergen exposure via human milk, although rare cases have been described of anaphylaxis (and more commonly, skin symptoms) to fish and cow milk due to antigens in human milk.

[3]Exclusively breastfed infants can develop eosinophilic gastrointestinal disorders (EGIDs), Food Protein-Induced Enterocolitis Syndrome (FPIES), or allergic proctocolitis, by exposure to food antigens through breastfeeding.

components in various populations, as well as the relative contributions of genetic and environmental influences on immune trajectories, growing evidence suggests links between early life environmental exposures and later health outcomes (17, 18).

Epidemiological evidence suggests the immune-system-enhancing role of a favorable gut microbial profile during the early months of life. For example, longitudinal cohort data suggest that infants at risk of developing asthma by the age of 3 y have transient gut microbial dysbiosis—specifically, reduced concentrations of 4 specific bacterial genera—during the first 100 d of life. Adding these 4 bacterial groups to germ-free mice decreased airway inflammation, suggesting a potential causal role of these microbes in asthma development (19). Other data suggest that calprotectin (calcium-binding proteins S100A8 and S100A9), which is present in high amounts in human milk, regulates inflammatory programming and cellular development during infancy. Deficiency of calprotectin was associated with an increased risk of newborns to develop unfavorable gut microbiota signatures and associated diseases (20).

During the panel's deliberations it was noted that differences observed between breastfed and formula-fed infants, such as gut microbial composition and metabolic profiles, are reduced after complementary foods are introduced, typically by the age of ~6 mo. For example, He and colleagues observed differences in serum metabolic profiles between breastfed and formula-fed infants (both a standard formula and a milk fat globule membrane isolate-supplemented experimental formula) starting from the age of 2 mo, which began to overlap by 6 mo and fully overlapped to the point of being mostly indistinguishable by the age of 12 mo (**Figure 1**). Further analysis indicated that this metabolic shift was driven by the introduction of complementary food (21).

Another analysis compared fecal microbiota and metabolome profiles at different ages among infants fed human milk or infant formula (22). In reporting the diet-associated variance in fecal metabolomics data at the age of 3, 6, 9, and 12 mo, the authors noted that effect of diet was no longer significant by the age of 12 mo (**Figure 2**) based on the study's measured endpoints.

Another study illustrated that T cell phenotype and function clustered separately at birth in preterm versus term infants, but converged at 40 wk postmenstrual age and were fully overlapping by 12 mo (**Figure 3**). The study also found that postmenstrual age exerted a greater influence than postnatal age on early T cell and microbiota development (23).

The panel considered whether the first 4 mo of life was the most appropriate time period for dietary interventions of bioactive ingredients with potential to modify the infant immune response. For example, early initiation (prior to the age of 4 mo) of complementary feeding is common among US infants (24); also, growth studies are required for infant formula approvals and these studies are required to enroll infants no older than the age of 2 wk and follow them for ≥15 wk (25). Nonetheless, the panel recommended the first 6 mo of life for this time period, based on data indicating that rates of exclusive breastfeeding (26) or formula-feeding drop around 6 mo in the USA, the age at which the Dietary Guidelines for Americans recommend introduction to complementary foods (2), which constitutes a major stimulus to the immune system (15).

Lastly, the panel determined that well-established surrogate markers of longer-term immune system effects are currently unknown. However, it recognized that tools are available to leverage multiomic data to identify such markers in the future.

Time period for assessment: First 12 mo of life.

The panel discussed that if major differences in immune system outcomes are not observed by the age of 12 mo, it is unlikely that such differences would be detected later in childhood. It concluded that 12 mo is an appropriate and feasible follow-up period for interventions examining the immune system effects of bioactive ingredients in infant formula. The panel suggested that longer-term follow-up assessments are not imperative for the regulatory determination of safety but can have value as later studies to examine, for example, microbiome development during the first few years of life (27, 28) or development of asthma, which would manifest later in childhood. It characterized longer-term assessments as exploratory and noted that availability of resources affects the feasibility of such assessments.

Using data on human milk composition and health outcomes of breastfed infants

The panel recognized the importance of identifying appropriate comparison groups when evaluating the immune system effects of bioactive ingredients in infant formula. Although the breastfed infant is an important reference group, the panel recognized a potentially broader range of normal immune responses in breastfed compared with formula-fed infants. Furthermore, human milk composition is influenced by factors that might also affect immune response, such as maternal genetics, physiological status, dietary intake (for some nutrients), and environmental

TABLE 2 Markers relevant to immune system development during early infancy

Markers reported to increase during infancy, unless otherwise noted[1]	Reference(s)	Markers reported as static during infancy[1]	Reference(s)
Innate			
—		Monocytes[4]	(17)
$\gamma\delta$ cells, %	(49, 68, 69)	—	
(TCR) $\alpha\beta$, double negative T	(48)	—	
(TCR) $\gamma\delta$, helper T[2]	(48)	—	
(TCR) $\gamma\delta$ cytotoxic T	(48)	—	
MR1T subpopulation of MAIT cell	(70)	MAIT cells	(71, 72)
—		NK cells[3,4]	(47, 48, 51, 73)
—		IL-6,[3,4] IL-1b, IFN-γ (small increases after some but not most toll-like receptor agonists in whole blood); IL-6 or IL-10 (no change after LPS stimulation of monocytes)	(74) (75)
Dendritic cell surface receptor HLA DR	(52)	—	
Polymorphonuclear leukocyte expression of membrane activated complex-1	(76)	—	
Adaptive			
—		Total T cells[3,4]	(47, 48, 51, 73)
—		Mature T cells[3]	(47, 48, 51, 73)
Terminally differentiated helper lymphocytes	(48)	Naïve helper T lymphocytes[4]	(47, 48, 77)
Terminally differentiated cytotoxic T lymphocytes	(48)	Cytotoxic T lymphocytes[3]	(47, 48, 51, 77)
Effector memory helper T lymphocytes	(48)	—	
Effector memory cytotoxic T cells	(48, 49)	Naïve cytotoxic T lymphocytes	(47, 48)
Memory helper T lymphocytes	(41, 48)	Central memory helper T lymphocytes[3]	(47–49)
—		Central memory cytotoxic T cells[3]	(47)
Activated and primed helper and cytotoxic T cells	(47)	—	
—		Recent thymic emigrants	(48, 71)
B cells[3]	(41, 47–50, 78)	Memory B cells[3]	(78)
Regulatory			
—		Regulatory T cells[3]	(48, 50)
—		Activated and activation primed T helper cells[3]	(47)
—		IL-2,[3,4] IL-4,[3,4] IL-5,[3] IL-10,[4] TNF-α,[4] INF-γ[3,4]	(79, 80)
Decreased IFN-γR1 on CD4, CD4+(N), CD4+(CM); Increased IFN-γR2 on CD4+, CD4+(N)	(81)	NSD IL-4Rα; IL-2Rα, IL-2Rβ on CD4 or subgroups; NSD IFN-γR1 on CD4+(EM); NSD IFN-γR2 on CD4+(CM), CD4+(EM)	(81)
TNF-α[4] response to LPS increased 2.5-fold (3 to 9 mo); TNF-α response to agonists decreased (2 wk to 6 mo)	(52, 82)	NSD in TNF-α production after Bacillus Calmette–Guerin; NSD in TNF-α after TLR 1–4 or PHA	(83, 84)
Linear increase in IL-2[3] post PHA (0 to 80 mo)	(85)	—	
—		IL-6,[3] IL-1b, IFN-γ[4] (small increases after some but not most toll-like receptor agonists in whole blood); IL-1b, IL-6, and IL-10 responses to most agonists were robust at birth and remained stable through 12 mo of age (whole blood and monocytes, respectively); IL-6 or IL-10[3] (no change after LPS stimulation of monocytes); NSD IL-6 after TLR1-4, PHA in monocytes; NSD IL-10, post-PHA over infancy in monocytes	(74)(86, 87) (75)(84)(85)

[1]All markers were assessed for change between the age of 0–3 mo and 6–12 mo; cytokine responses to agonists are reported where specified. MAIT, mucosal-associated invariant T cells; MR1T, MR1-restricted T; NK, natural killer; NSD, no significant difference; PHA, phytohemagglutinin; TCR, T cell receptor; TGF, transforming growth factor; TLR, toll receptor.

[2]Decreased during infancy.

[3]No significant difference between breastfed and formula-fed infants, see **Supplemental Table 2**.

[4]Difference between breastfed and formula-fed infants, see Supplemental Table 2.

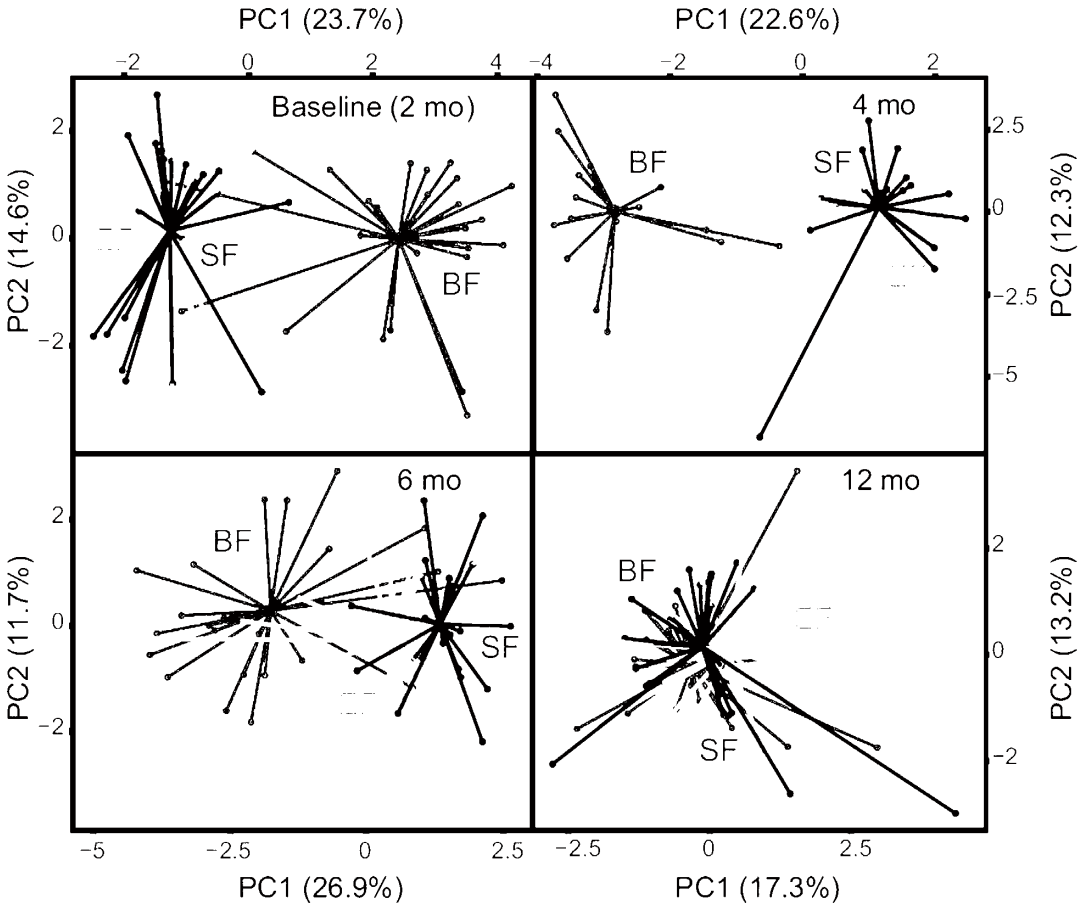

FIGURE 1 Differences in serum metabolic profiles of breastfed (BF), standard formula-fed (SF), or experimental formula-fed (EF) infants at the age of 2, 4, 6, and 12 mo. PC, principal component analysis of serum metabolite concentration data. Reproduced with permission from reference 21. Creative Commons license: http://creativecommons.org/licenses/by/4.0/.

exposures (29). Therefore, the panel determined that it is ideal to compare infants—in the context of an RCT—fed a standard infant formula (control group) to infants fed the same infant formula with the addition of the bioactive of interest (experimental group). The panel also suggested that a reference group of concurrently enrolled breastfed infants would provide a meaningful biological comparison, generate normative data for breastfed infants, and allow for adjustment of seasonal infections.

The panel agreed that human milk substances have more a priori safety compared with bioactive substances not found in human milk, and that human milk composition is a reasonable guide for informing the amounts of bioactives added to infant formula. Risks of adverse events or analytical abnormalities are expected to be less likely for substances found in human milk compared with those not found in human milk. The panel was aware of a few examples of substances proposed for addition to infant formula that are not found in all maternal milk [e.g., fucosylated human milk oligosaccharides (HMOs) not present in milk of nonsecretor/fucosyltransferase 2 mothers; other pre- and probiotics].

Even with human milk composition as a guide for appropriate concentrations of bioactive substances to be added to infant formula, identifying these concentrations is challenging because the composition of human milk is complex and dynamic, and available data are limited by suboptimal collection methods and use of analytical assays that have not been validated for human milk. Moreover, little data exist on normal ranges of immunological substances in human milk among healthy women in different populations (30, 31), although one study identified a common but relatively small set of Igs, cytokines, chemokines, and growth factors present in mature milk produced by healthy women independent of their geographical location (32). The panel was not charged to provide concentrations of bioactive substances to be added to infant formula (and did not take a position on this issue), but to recommend assessments that demonstrate the safety of bioactive ingredients in whatever concentrations are used. Nonetheless, the panel suggests considering evaluation of bioactives at concentrations of feeding that reflect mean concentrations measured in human milk, as well as at the highest concentrations (i.e., 95th percentile) measured in human milk, noting that animal studies may be needed for feeding at multiples of human milk exposures.

Human milk bioactives vary in their mechanisms of action for influencing infant immune outcomes—some have direct implications for infant immunity (e.g., Igs that are absorbed), others may trigger or suppress the development and maintenance

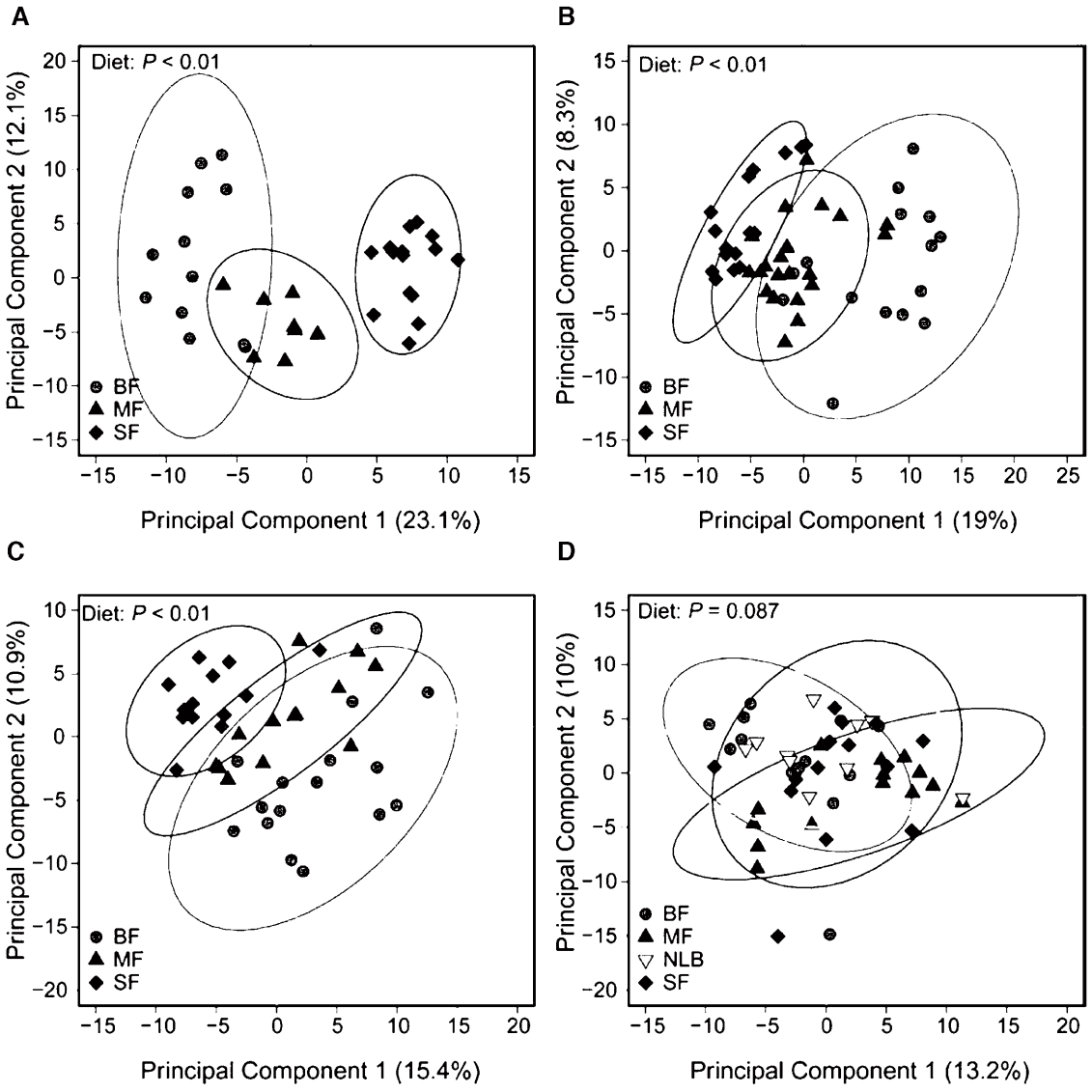

FIGURE 2 Visualization of variance associated with infant diet in fecal metabolomics data from infants consuming differential diets at the age of 3 (quadrant A), 6 (B), 9 (C), and 12 (D) mo. BF, breastfed; MF, cow milk formula-fed; NLB, no longer breastfeeding; SF, soy-based formula-fed. $N = 10$–20 per group and age. Reproduced from reference 22, by permission of Oxford University Press.

of the infant's immune profile, and still others may indirectly affect the infant immune system via gut microbial composition and its effects on what gets absorbed from the gut as well as immune responses. The current literature is limited with respect to isolating the relative contribution of individual bioactive ingredients to infant immune outcomes in humans, let alone elucidating the mechanism of action of any observed effects. Few human observational studies of the relations between breastfeeding and infant health report specific immune system outcomes, and the few RCTs that exist tend to examine human milk overall rather than its singular components (33). Furthermore, individual bioactive ingredients may have differential effects in their natural milk milieu versus in another environment or matrix.

A recent article suggested that human milk composition and its resulting biological function is greater than the sum of its parts and called for critical advancement in the study of human milk as a biological system. This included mapping various nutritional and bioactive components of milk by different stages of lactation and their function and mechanism of action for promoting infant growth, development, and survival (34).

Addressing complexities associated with many bioactive ingredients of interest

The panel acknowledged that complexity of bioactive ingredients and their production methods may affect biological activity and safety, but sought immune system safety endpoints that would be appropriate whether, for example, an ingredient is derived from a bovine or human recombinant form or goes through different processing methods in preparation for food use. GRAS notifications must characterize the nature of an ingredient and its production method(s). If a different form or processing

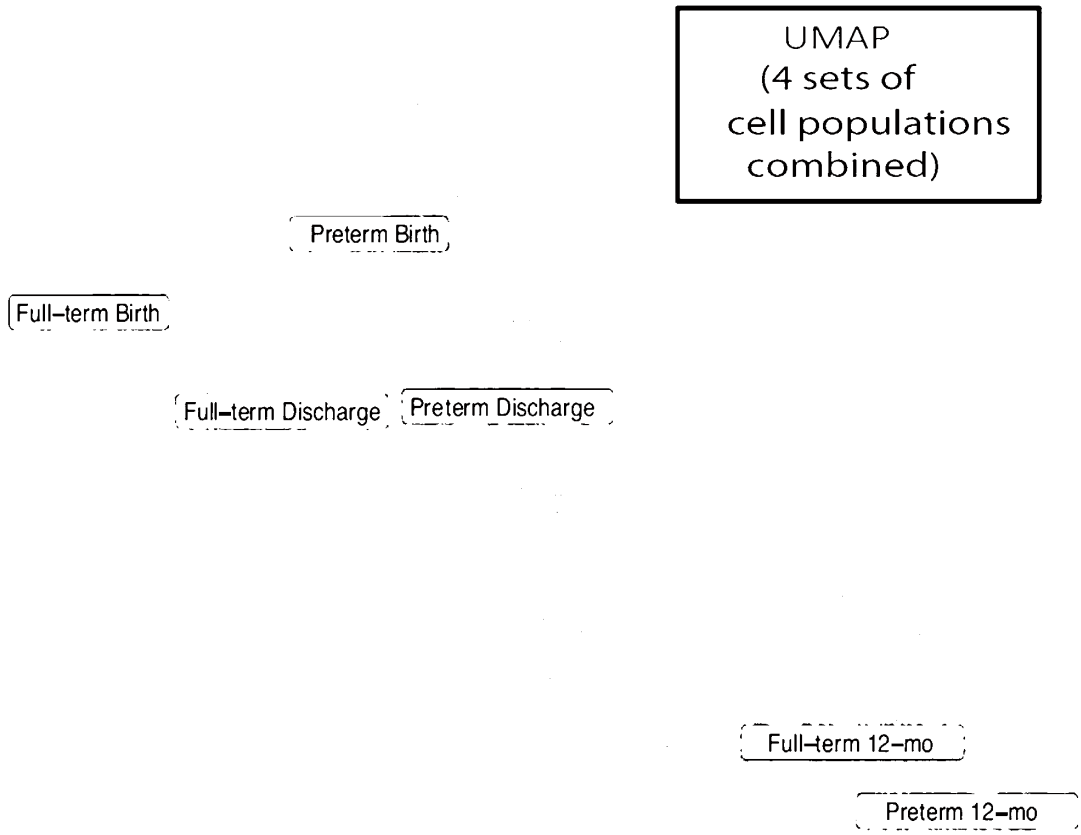

FIGURE 3 Uniform manifold approximation projection (UMAP) visualizing convergence of T cell phenotype and function between groups of preterm and term infants. Reproduced with permission from reference 23. Creative Commons license: https://creativecommons.org/licenses/by-nc-nd/4.0/.

method for the ingredient is proposed, a separate GRAS notice is typically submitted.

The panel also recognized that many immunologically active substances are produced endogenously, that some potentially human milk bioactive components are produced during the digestion of human milk proteins (35), and that activity of a substance consumed orally depends on digestive physiology and bioavailability. Characterization of an ingredient and consideration of its digestive breakdown products includes absorption, distribution, metabolism, and excretion, which are routinely evaluated in the GRAS process.

Identifying suitable markers of developmental immune competence

Recognizing that the major advances in understanding of human immune ontogeny over the past 1–2 decades have illuminated the complexity of immune development, the panel anticipated that it would identify multiple markers that (considered together) could indicate developmental immune competence.

Markers with established relations to disease or adverse health outcomes are desirable, but the emerging nature of discoveries related to the trajectory of immune development is associated with a lack of normal values or reference ranges for many markers of immune development. Therefore, it is unknown what, if any, disease associations may exist when values are within age-appropriate ranges reported for healthy infants. A statistically significant difference between experimental and control groups is necessary, but insufficient, to infer an important biological difference in health outcomes. In addition, environmental influences such as birth delivery mode and maternal and infant antibiotic exposure may affect certain markers. Collecting these variables could help determine whether stratification of subjects by those environmental characteristics is needed. The panel agreed to limit its recommended safety assessments to endpoints with established reference ranges. It also agreed that clinical observations of adverse events, confirmed by validated methodologies, are clear indicators of safety.

The panel also discussed the importance of the availability of standardized, reproducible assays for any markers it recommended. It considered the availability of widespread laboratory capacity to implement potential assessments, recognizing a lack of standardized methods and reference ranges for infants in assessing the microbiome and metabolome, and for laboratory execution of multiplex immunoassays.

Developing comprehensive and pragmatic recommendations

The panel aimed to develop practical, usable recommendations for stakeholders in regulatory, industry, and academic settings. It recognized the proliferation of tools and technologies to assess a myriad of clinical, immunological, and vaccine-related endpoints of interest, but agreed that it would be most useful to identify a core set of standardized markers (with accepted normal values or reference ranges) that are both sufficient for demonstrating safety and feasible for GRAS petitioners to produce. The panel concluded that recommendations for markers without reference ranges would be helpful to designate as additional, optional assessments, with the hope that their collection will inform the future development of reference ranges.

Recommended Endpoints for Assessing the Safety of Novel Bioactive Ingredients in Infant Formula

The panel's recommended safety assessments, presented in Tables 1 and 2, are based on outcomes of its discussions of the topics in the section titled Key Considerations to Inform Development of Recommended Endpoints. These outcomes are summarized in **Box 4**.

Box 4:

Guiding Criteria: Recommended Safety Assessments for Bioactive Ingredients in Infant Formula

- Help facilitate a regulatory decision about whether a new ingredient proposed for use in infant formula is safe (i.e., poses reasonable certainty of no harm).
- Occur during the first 12 mo of life and are based on dietary interventions of bioactive ingredients conducted during the first 6 mo of life.
- Occur in the context of a blinded RCT that compares infants fed a standard infant formula (control group) to infants fed the same infant formula with the addition of the bioactives of interest (experimental group).
- Include a reference group of concurrently enrolled breastfed infants to provide a meaningful biological comparison, generate normative data for breastfed infants, and allow for adjustment of seasonal infections.
- Limited to markers with established normal values or reference ranges, which can be used to identify abnormal or disease states.
- Remain consistent regardless of a bioactive ingredient's source or processing methods.
- Are practical and feasible for stakeholders in regulatory, industry, and academic settings.

The assessments are divided into recommended standard clinical endpoints to demonstrate safety for immune-related outcomes in infants from birth to the age of 6 mo (Table 1) and a listing of candidate markers relevant to immune system development during infancy (i.e., first 12 mo of life) (Table 2).

Recommended standard clinical endpoints to demonstrate safety for immune-related outcomes in infants from birth to the age of 6 mo

The endpoints in Table 1 are collectively intended to represent the complex spectrum of symptomatology that could indicate development of immunologically mediated hypersensitivity to ingested food ingredients. Therefore, when evaluated collectively during the first 6 mo of life, the panel concluded that these endpoints are sufficient to demonstrate safety of novel bioactives in infant formula for immune-related outcomes in infants. Whether the endpoints are evaluated as primary or secondary endpoints depends on the hypothesized effect(s) of the particular novel bioactive substance under study.

Table 1 includes endpoints related to growth and growth velocity, infection and other adverse effects related to inflammation, and allergy and atopic diseases. These endpoints are similar to markers that a previous expert group identified as particularly useful (according to clinical relevance and involvement of immune functions) for examining immune modulation by dietary ingredients (36). Vaccine response is included in Table 1, as low vaccine response and frequent infections have been linked to infant immune deviation from normal (37). Vaccine response is more comprehensive than responses to cytokines or other isolated measures (38). Maternal and infant factors that potentially influence vaccine response can be controlled in RCTs and can be characterized for reference breastfed infants (39, 40). Notwithstanding that blood collection is implied for vaccine response, which may be a barrier for recruitment of study participants, the panel found the integrated immune response to vaccination to be a meaningful and reliable measure of the infant immune system.

The evidence base for Table 1 draws from studies registered at www.clinicaltrials.gov. To add to this evidence base, cohort and observational studies may be considered, but RCTs are preferred, using PICO (Population/problem, Intervention, Comparison, and Outcome) methodology and powered appropriately for the primary outcome used to assess safety.

Although outcomes related to other biological systems were not the focus, the principles used to identify the endpoints in Table 1—such as predictive value for health outcomes and identification of metabolomics and microbiome measures as covariates—are relatively generalizable to other systems and provide a starting point to help identify additional clinical signs that would encompass a broader group of outcomes.

Markers relevant to immune system development during early infancy

Starting with markers of immune function described by previous expert groups (10, 11), the panel compiled information from peer-reviewed literature published since 2002 relevant to immune system development during early infancy. The resulting list (Table 2) identifies innate, adaptive, and regulatory immune system markers for which quantitative measurements in healthy term infants during the first 12 mo of life have been reported (and in some cases, illustrated graphically). The markers are grouped into those for which evidence indicates occurrence of specific and meaningful change in absolute or relative concentrations over time during infancy (column 1) and those for which no

such change in concentrations has been indicated (column 3). Only the direction of change is reported in Table 2; the panel pointed out that reported ranges of absolute and relative values for the markers between studies are dependent on methods used to assay those markers. Furthermore, these reported ranges are not reference ranges and cannot necessarily be used to identify abnormal or disease states.

Therefore, in the context of safety assessment for bioactive ingredients in infant formula, the markers in Table 2 are to be viewed as examples of indicators that an infant is following a stereotypical pattern of immune development during the first year of life, not as a comprehensive set of recommended standard immune system safety endpoints. The panel emphasized that study investigators (in consultation with the ingredient manufacturer) should determine which immune system tests are necessary based on a thorough analysis of the potential, hypothesized effect(s) of the new bioactive ingredient and also on preclinical data (10). The panel also noted that the markers in Table 2 are not exhaustive and that even if typical values were reported for all, absence of immunopathy is not guaranteed.

Immune markers are influenced by numerous environmental factors, such as geography; mode of birth delivery; feeding (breastfed or formula-fed); exposure to household pets and environmental chemicals and pathogens; antibiotics and other medications; and recent illness; as well as biological factors including genetics, sex, and ethnicity (39–52). Furthermore, microbiome measures during infancy are responsive to nutrition and may mediate immune development (53). Except where noted, the studies in Table 2 did not take feeding (nor other environmental factors that could influence concentrations of immune markers) into account. Studies on feeding are summarized in Supplemental Table 2 and indicate that consistent differences between breastfed and formula-fed infants in markers of immune system development have not been established.

The panel noted that immune markers reported as unchanged during infancy could respond to a nutritional intervention. However, because rapidly changing physiologic states are most sensitive to nutritional effects (54–56), such effects are more likely to be detected by evaluating immune markers with evidence of maturational change during infancy.

The panel also discussed study design features to improve the usefulness of immune markers during infancy. As stated in **Box 4**, these markers should be evaluated in the context of an RCT that compares infants fed a standard infant formula (control group) to infants fed the same infant formula containing the bioactive(s) (experimental group). Breastfed infants should be concurrently studied as a reference group. The sensitivity of particular markers to a bioactive ingredient is likely improved where an effect of feeding has been established by a difference between breastfed and formula-fed infants. Other important study design features include recording information about factors reported to influence results (e.g., sex, antibiotics, mode of birth) and allowing assessment of potential subgroup differences; including culturally diverse populations of infants; collecting specimens at multiple timepoints, including at baseline, in order to better understand any changes observed within study subjects; and focusing outcome comparisons among study groups, rather than external values. Biobanking of study specimens is critical to enable future analysis of additional immune parameters as more sophisticated analytic tools and capabilities are developed.

The panel recognized the potential challenge of recruiting study populations of exclusively formula-fed infants given that only 16% of US infants have never been breastfed (4). It was also aware that blood collections may be a barrier to recruitment, but recognized that advances in analytical techniques allow extremely small samples of blood to be used for examining complex immune system interrelations. The panel encourages exploration of creative study designs and novel collection/analytic techniques that use blood routinely obtained for other purposes (e.g., assessment of infant iron status) or use alternative compartments such as sweat, saliva, stool, or urine.

The contents of Tables 1 and 2 represent available evidence, although new evidence from the rapidly expanding fields of infant immune development and ontogeny may warrant future updates. **Box 5** summarizes the expert panel's added contributions to prior efforts related to evaluating the effects of new infant formula ingredients on immune system development.

Box 5:

Expert Panel Contributions to the Evaluation of Effects of Ingredients on Infant Immune System Development

- Added details to recommended clinical observations.
- Added response to vaccination as a recommended assessment.
- Called out stereotypic immune system development and convergence of immune, metabolic, and microbiome markers to focus the observation period.
- Summarized evidence on changes in immune markers during infancy for innate, adaptive, and regulatory compartments, where evidence is not yet sufficient to use as standard markers.
- Identified research gaps, variables to control, and emerging science opportunities.

Research Gaps

The panel's presentations and discussions brought to light a list of research gaps, summarized in **Box 6**. Research is underway to explore many of these areas, but much remains unknown.

Box 6:

Research Gaps Related to Assessing the Safety of Bioactive Ingredients in Infant Formula

- Reference ranges for markers of immune development.
- Longitudinal data to examine potential immunomodulatory effects.
- Reference ranges for immunomodulatory substances in human milk from diverse populations.
- Examination of the effects of specific bioactive substances in human milk on neonatal immune development and infectious/allergic disease outcomes.
- Additional information about differences in health outcomes between breastfed and formula-fed infants.

- Improved resolution of the ontogeny of infant immune development.
- Better understanding of microbiome markers of normal immune system development and definition of dysbiosis.
- Data to enable the use of systems biology approaches to evaluate safety and efficacy of bioactives in infant formula.

Reference ranges for markers of immune development

The panel agreed that development of age-specific reference ranges for markers of immune development is of high importance; lack of such ranges precluded the panel from including specific markers of immune development among its recommended standard safety endpoints. Reference ranges developed from diverse populations at various time points during the first year of life (e.g., the age of 2, 4, 6, and 12 mo) are desirable. Many immune phenotypes and functions undergo substantial change during that time, confounding the assessment of nutritional effects. Age-specific reference ranges must be established in order to measure potential short- and long-term effects of nutrition interventions. Additionally, postmenstrual age rather than postnatal age (i.e., calendar age or days of life) may exert greater influence on immune markers (23) and has not typically been determined in normative studies. Age-specific reference ranges will inform whether measured outcomes indicate normal, safe, and/or efficacious responses to nutritional interventions with the potential to modulate immune response. They will also help researchers determine which fold changes or thresholds are clinically meaningful.

Longitudinal data to examine potential immunomodulatory effects

Although the panel's recommended assessment period for intervention effects is the first 12 mo of life, longitudinal research to examine persistence of any differences in immune response would help indicate potential programming effects. Sophisticated methods that could detect whether such effects could be occurring during the intervention are needed.

Reference ranges for immunomodulatory substances in human milk from diverse populations

Few studies have examined natural variations in the immunological substances of human milk among healthy women living in different geographic, dietary, and socioeconomic settings. In 1 example, substantial variation was measured within and among human subpopulations with regard to immune factors in milk produced by healthy mothers in high-, middle-, and low-income countries. Only 9 of the 23 analyzed factors were detected in all or most of the samples collected in each population (32). In addition, a multicenter cohort project in 4 countries is underway to establish reference values for micronutrients and macronutrients in the milk of well-nourished women; samples obtained will also be used to perform analyses of human milk oligosaccharides and proteins (31). Data from this study and others that examine human milk composition will further understanding of typical ranges of immunological substances in human milk and suggest how those ranges might vary by geography, environment, maternal lifestyle, and other variables. Such understanding would inform concentrations of bioactive substances to be added to infant formulas intended for different populations.

Examination of the effects of specific bioactive substances in human milk on neonatal immune development and infectious/allergic disease outcomes.

Studies designed to clarify the relative contribution of individual bioactive ingredients to infant immune development and outcomes in humans are limited, and few human observational studies of the relations between breastfeeding and infant health report specific immune system outcomes. Linking data on milk composition with maternal metadata and infant outcomes can help clarify, for example, how specific cytokine or human milk oligosaccharide profiles are linked to specific physiological/immune responses, or how bioactive substances could work together to potentiate or inhibit immune responses.

It is also of interest to better understand how the physiological effects of a single bioactive substance are impacted by the entire human milk matrix. For example, are the substance's effects altered or modified by other substances in the matrix, or are there functional or physical interactions within the matrix of human milk that are not present in infant formula? This could help predict potential differential effects of a substance's effects in human milk compared with its effects in infant formula. Differences in health outcomes (e.g., diarrhea, respiratory infections) between breastfed and formula-fed infants tend to be more pronounced in populations with a higher prevalence of those adverse outcomes.

Additional information about differences in health outcomes between breastfed and formula-fed infants.

Differences in certain health outcomes and in metabolic and metabolomics profiles between breastfed and formula-fed infants appear to converge around the period of complementary food introduction, but the panel recognized that persistent differences may still remain unmeasured, and the pathways leading to those outcomes unknown. For example, differences in microbiome characteristics may exist, but methods to assess potential differences need further study. Similarly, consistent differences in markers of immune system development between breastfed and formula-fed infants have not been established. Future studies that control for various environmental variables reported to independently affect immune markers (e.g., mode of birth and antibiotic exposure along with method of feeding) may be able to demonstrate such differences, if they exist.

Improved resolution of the ontogeny of infant immune development.

Infant immune development is characterized as a continuum, but further study is needed to clarify the ages at which various functions stabilize at what are considered adult concentrations. Standardized methodologies to determine the age of stabilization

for various functions would be helpful, and these ages might vary by population characteristics and environmental variables. Such understanding would inform the timing of nutritional interventions for optimal immunomodulatory outcomes.

Better understanding of microbiome markers of normal immune system development and definition of dysbiosis.

Dysbiosis is a term increasingly referenced in the scientific literature (57). Despite growing interest in its role in both acute and potentially chronic disease outcomes (58), a definition for dysbiosis has been elusive. Because there is a lack of consensus on what constitutes a healthy microbiome, it is not clear how to define one that is unhealthy, due to the dynamic nature of the gut microbiota complicating the process. Dysbiosis can be characterized by a loss of beneficial microorganisms, an increase in harmful microorganisms, or a loss of overall microbial diversity. Research that identifies links between specific microbial states and clinical outcomes is needed.

Data to enable the use of systems biology approaches to evaluate safety and efficacy of bioactives in infant formula.

As systems biology approaches integrate data about the microbiome, metabolomics, proteomics, and other fields, they can help characterize a baseline for the progression of immune response and illuminate why responses might differ between populations and by certain population characteristics. Specifically, these approaches could be used to better understand the effects of bioactive substances in human milk and infant formula by combining results from studies of different feeding methods and using artificial intelligence and/or machine learning approaches to look for patterns of markers that vary by diet. With respect to genomics, variants or mutations in certain genes could dictate major differences in response to a nutritional intervention. Systems biology approaches could help to identify any populations that might be at risk from consuming a certain bioactive substance.

Although systems biology approaches are resource-intensive and still relatively emergent, the panel recognized their potential to synthesize information from multiple disciplines in a way that is useful for both research and regulatory objectives. To maximize the potential of systems immunology, a highly standardized approach to the various clinical, technological, and bioinformatics components is important, as is the inclusion of appropriate immunological expertise (59). Advancement of tools for data analysis, interpretation, and visualization is also important for analyzing vast quantities of data and translating the results in order to achieve meaningful outcomes. In the meantime, the panel encourages researchers to bank small serum/plasma samples to be evaluated for additional parameters in the future.

Application of the Expert Panel's Findings and Recommendations

The expert panel's findings and recommendations (i.e., **Box 4**, Tables 1 and 2) are suitable for immediate application by stakeholders in industry, regulatory, and academic settings. In industry, they can guide the design of clinical trials conducted to demonstrate the safety and/or efficacy of novel bioactive ingredients in infant formula on immune development and function. In regulatory settings, they provide further guidance to FDA officials and future GRAS panels charged with reviewing data from petitioners to demonstrate the safety of these ingredients in infant formula. In academia, they inform research priorities, with an emphasis on the need for reference ranges for immune system markers that indicate healthy immune system development.

The findings and recommendations are also applicable beyond their original purpose for assessing the safety of bioactive ingredients in infant formula. They may be used by future expert panels as a basis for recommendations on assessing the safety of medical foods or foods for special dietary uses, whether intended for infants or other populations. They may also inform future recommendations for safety assessment of immunomodulatory ingredients in foods other than infant formula; this is particularly timely given the expected growth in the market for food products that claim to support immune function (60).

The expert panel carried out its charge with the US regulatory system in mind, but regulatory processes in the USA can influence such processes in other countries. If applied in non-US settings, stakeholders should consider how the findings and recommendations may need to be tailored to the population of interest, given potential population-based differences in baseline measures and the relative feasibility of implementing the recommendations in other settings.

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