TITLE:

Factors Influencing Gastrointestinal Tract and Microbiota Immune Interaction in Preterm Infants.

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ABSTRACT

The role of microbial colonization is indispensable for keeping a balanced immune response in life. However, the events that regulate the establishment of the microbiota, their timing, and the way in which they interact with the host are not yet fully understood. Factors such as gestational age, mode of delivery, environment, hygienic measures and diet influence the establishment of microbiota in the perinatal period. Environmental microbes constitute the most important group of exogenous stimuli in this critical time frame. However, the settlement of a stable gut microbiota in preterm infants is delayed compared to term infants. Preterm infants have an immature gastrointestinal tract and immune system which predisposes to infectious morbidity. Neonatal microbial dynamics and alterations in early gut microbiota may precede and/or predispose to diseases such as necrotizing enterocolitis, late-onset sepsis or others. During this critical period, nutrition is the principal contributor for immunological and metabolic development, and microbiological programming. Breast milk is a known source of molecules that act synergistically to protect the gut barrier and enhance the maturation of the gut-related immune response. Host-microbe interactions in preterm infants and the protective role of diet focused on breast milk impact are beginning to be unveiled.

Introduction

Current evidence supports the role of microbiota in promoting and maintaining a balanced immune response and the establishment of the gut barrier in the immediate postnatal life. The human body harbors a dynamic and complex microbial population, which includes around 500-1000 different species. In the perinatal period neonates are exposed not only to a vast microbial diversity but also to a variety of organisms such as viruses, fungi and parasites. After weaning, the infant's gut is colonized by a rapidly diversifying microbiota that leads to an adult-like pattern of intestinal flora. The composition of the neonatal microbiota is influenced by numerous perinatal factors such as mode of delivery, environment, hygienic measures, antibiotic treatment and breastfeeding practices (1). Adverse circumstances affecting the establishment of a normal microbiota may result in failure in the development of a balanced immune response but also have a significant impact on the intestinal mucosal barrier function and intestinal maturation. Furthermore, these disturbances may predispose to specific diseases later in life. Early host-microbe interactions may provide a signal for immune system development and maturation (2).

The "Early programming" theory is based upon the fact that environmental exposures including nutritional during the perinatal period can induce permanent changes in many physiological processes. Postnatal diet mainly breastfeeding practices are therefore critical for the ongoing developmental maturation of many organ systems and optimal physiological functions The effects of early programming extend into adulthood and are linked to the risk of acquiring specific diseases in adult life (3-5).

Prematurity and microbiota

In the last decades, mortality of very preterm infants has been substantially reduced. Improvement in the survival rates has been attributed to a series of factors such as

regionalization, generalization of antenatal steroids, new modalities of mechanical ventilation, and the use of surfactant replacement therapy (6). However, this has not always been paralleled by a simultaneous decrease in morbidity. The prevalence of serious complications such as retinopathy of prematurity, bronchopulmonary dysplasia or periventricular leukomalacia has remained practically unchanged especially in the very preterm infants (<32 weeks' gestation) (7). Of note, mortality as a consequence of neonatal septicemia has been reported to be around 18% of all neonatal deaths. Interestingly, approximately 50% of these deaths occur in the first week of life, and the remaining 50% are nosocomial infections acquired during prolonged hospitalization (8).

The role of microbiota in preterm birth and the consequences of prematurity upon postnatal microbiome development are emerging fields of discussion. The exact mechanisms responsible for preterm birth are multifactorial and yet not completely unveiled (table 1). However, the link between infection and prematurity has been well established. In addition to the traditionally accepted ascending infection from the lower genital tract recent research suggests that other forms of bacterial spreading could be responsible for preterm delivery (9). Interestingly, studies by Madianos et al (10) and Aargard et al (11) have shown resemblances between fetal and neonatal bacterial colonization and maternal oral cavity and placental flora strongly suggesting a hematogenous dissemination in the perinatal period.

The interaction of preterm infants' altered gut microbiota with an immature immunologic intestinal response triggers pro-inflammatory and counter-inflammatory cytokine response (12). These factors are extremely important for preterm infants because they influence the pattern of bacterial gut colonization, which is different to that in the healthy full-term infant (figure 1). Briefly, preterm infants showed retarded *Bifidobacterium*- microbiota colonization, a high prevalence of *Staphylococcaceae*,

Enterobacteriaceae, *Enterococcacea*e and other lactic acid bacteria as the genus *Lactobacillus* and *Weissella* in a low-diversity bacterial ecosystem (13, 14, 15). In preterm birth a negative correlation with gestational age and a tendency toward a higher inflammatory response has been associated with higher presence of *Enterobacter*, *Enterococcus*, *Lactobacillus*, *Photorhabdus*, and *Tannerella* spp. (17). Microbial colonization of meconium and feces of preterm infants analyzed during the first months of life using traditional culture-based methods and molecular techniques to analyze bacterial diversity revealed that Firmicutes was the predominant phylum in meconium while Proteobacteria was present in neonatal fecal samples. Interestingly, the Proteobacteria phylum may increase the inflammatory response in the preterm intestine, and it has been speculated that exposure to low quantities of lipopolysaccharides (LPS), flagellin or other Gram negative substances may interact with the intestinal Toll Like Receptors (TLR) and confer "tolerance" to further inflammatory stimuli (18). In a recent study, it was shown that early Proteobacteria colonization might cause an exacerbated immune response, which may impact on the intestinal barrier enhancing bacterial translocation and the risk to develop sepsis, NEC and other inflammatory problems (19, 20, 21). The concept of bacterial translocation and gut-originated sepsis and NEC as a cause of systemic infectious complications has emerged over the last years, although the exact clinical relevance of these phenomena continues to be debated (21).

Gastrointestinal tract immune system and microbiome interaction in preterm infants

At birth, the adaptive immune system is quite rudimentary and untrained. Hence, normal term newborns highly rely on trans-placental passage of mother's IgG during the last three months of gestation for control of pathogens showing a remarkable

activation of T cells (22, 23). Furthermore, the immune response to gut pathogens in term newborn infants still requires protein components from mother's milk to efficiently fulfill TLR binding and signal transduction (24). Innate immune functions in the fetus are ontologically regulated through gestation; hence, developmental events may give rise to differences in the innate immune response of the neonate, and particularly in premature newborns, putting them at high risk of developing severe infections. A high proportion of Th1-polarizing cytokines during pregnancy has been related to a high probability of abortion (25). Therefore, in the fetus, expression of Th2 cytokines may be the strategy to avoid negative reactions. However, premature birth renders the newborn more susceptible to infections (22).

Nevertheless, preterm infants have a substantially reduced trans-placental transfer of maternal antibodies and an immature innate immune system. Both these circumstances facilitate the preterm to acquire infections (23, 24). Shorter gestational duration is associated with reduced proportions of peripheral blood lymphocytes (26), pro-inflammatory cytokine response and secretion of antibacterial peptides, smaller counts of phagocytic peripheral monocytes and reduced leukocyte endothelial transmigration (27, 28). The reduced synthesis of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, etc., in response to bacterial endotoxin lipopolysaccharide (LPS) binding to TLR4 does not depend on the low expression of TLR4 and CD14 but to a yet unknown factor downstream in the signal transduction pathway (29). Transmigration of neutrophils in innate immunity is an essential process for immune cell recruitment towards inflamed tissues. It is a highly regulated process that involves a series of adhesion steps to specific molecules comprising an initial "rolling" of neutrophils on the endothelial surface, followed by firm adhesion, and finally transmigration and exit from the blood vessel. Therefore, this process is favored by Th1-polarizing cytokines (pro-

inflammatory); however, the fetus follows a Th2-polarization strategy that affects the efficiency of transmigration (22). Finally, there is a likely reduction in phagocytic activity by monocytes, with respect to term newborns (26, 30), but also of gut dendritic cells (31). These cells ultimately eliminate bacteria and therefore constitute an essential component of the innate immune system; however, they also intervene in the process of antigen presentation, which constitutes the initial step in the adaptive immune response.

Abnormal patterns of colonizing gut bacteria in infants with an immature host innate and adaptive immune system will accelerate de advent of infectious diseases, particularly when catheters and enteric feeding facilitate pathogenic invasion. The remarkable importance of maintenance of immune homeostasis in preterm infants derives from the fact that, although clinical and pharmacological approaches have decreased mortality of infected preterm infants, these processes cause a high pro-inflammatory and pro-oxidant stress that inevitably leads to irreversible damage to vital organs, including brain and intestine that often results in neurodevelopment impairment (32) and gut bacteria over-reactivity that may lead to IBD (33).

Sepsis and microbiome

Sepsis remains one of the most common causes of neonatal morbidity and mortality in the preterm population (8, 34). Early-onset sepsis occurs in 1.5 to 1.9% of very low birth weight (VLBW) and late-onset sepsis (LOS) in 20%, with mortality altogether approaching 18% (8) and a higher risk of cerebral palsy in preterm infants (33). Gram-positive organisms are the most prevalent microbes in LOS, and among them Coagulase-negative *Staphylococcus* (CONS) are the most common microbes followed by specific gram-negative bacteria (8). However, it has been shown that a dysbiosis in preterm microbiota composition but not an increased prevalence of potential pathogens is associated with sepsis (19). Thus, the presence of *Enterobacter* and *Staphylococcus*

spp. has been associated with NEC and sepsis, respectively (34). It has been also described that lower bacterial diversity present in meconium samples from preterm infants is related to higher risk of sepsis (35). Our results in very low birth weight (VLBW) preterm twins showed higher levels of *Enterobacteriaceae* family and lower levels of *Bifidobacterium* spp in the sepsis neonates as those observed in their healthy twin controls. By pyrosequencing, we also found a high presence of Proteobacteria phylum (*Enterobacteriaceae* family) in septic infants. Principal Coordinate Analysis (PCoA) showed differences between sepsis and control groups although microbial profiles were twin-pair and neonate-dependent.

Necrotizing enterocolitis and microbiome

Necrotizing enterocolitis (NEC) has become the one of the most severe and dreaded diseases seen in neonatal intensive care (36). In North America, it affects approximately 7% of babies weighing between 500-1500 grams and approximately 20 to 30% of these babies die of this disease (37). Sequels are not limited to the gastrointestinal system, where short gut is a common problem, but severe neurodevelopmental delays are commonly seen especially after NEC that requires surgery (38). The initiation of early enteral feeding in preterm infants favors an earlier achievement of full enteral feeding and does not increase the risk of NEC; however, the optimal advance of enteral feedings remains a debatable question that ongoing studies intend to clarify (39).

The pathophysiology of the most common form of this disease seen in preterm infants has remained elusive; however, several factors predisposing to the development of this disease have been identified. Functional immaturity of the gut likely plays a role (36). The fact that the disease often occurs in clusters has suggested a microbial etiology. As of yet, no single microbe has been found that is causative, but an inappropriate colonization prior to the development of the disease has been suggested (40).

Studies of the microbial ecology prior to the development of NEC using sequencing technology show differences in those babies who develop the disease versus matched controls (40, 41). Preliminary studies on fecal microbiota from unaffected preterm infants and from infants with NEC both prior to and during NEC showed an association between intestinal microbial species and NEC. A matched analysis of predominant phyla prior to the development of the disease shows that Proteobacteria or the relative proportion of Proteobacteria with the other phyla may be involved. The Proteobacteria phylum contains numerous gram-negative genera such as *Klebsiella* and *Escherichia coli*, which may be pathogenic (42, 43).

Promising approaches to prevent NEC are directed towards the inhibition of TLR4. In experimental models the molecule C34, a 2-acetamidopyranoside (MW389) (44) and amniotic fluid (45) have reduced the incidence of experimental NEC. Epithelial Growth Factor (EGF) (46) and hepatocyte growth factors (HGF) (47) were found to be the mediators of amniotic fluid derived protection. An improved understanding of the factors influencing the development of the microbiota or that modulate its composition may offer new strategies, tools, and opportunities for preterm interventions that reduce the risk of specific diseases as NEC.

Potential tools to modulate preterm microbiome

Among the different strategies employed to reduce the incidence and/or severity of NEC and prevent LOS in preterm infants, the use of human breast milk (HM) and/or supplementation with pro-and/or-prebiotics have rendered effective results (48, 49, 50). In the following paragraphs we will expand on these strategies.

i) *Human Breast milk as gold standard diet for preterm*

HM is considered the gold standard for infant nutrition and constitutes the main postnatal link between mother and infant. HM contains bioactive components that

directly influence the developing infant and shape the development of the intestinal microbiota. Beyond the nutritional composition, it contains several immune-related substances such as regulatory cytokines and growth factors, which are considered protective and stimulate the immune system with a positive impact on health. In addition, it contains other active non-specific factors, such as lysozyme, lactoferrin, oligosaccharides and also, microorganisms, which also contribute to enhance anti-infective and immune-modulatory properties (51). The implication of HM in both prevention and treatment of NEC has long been recognized; however the specific compounds responsible for these beneficial effects are yet unknown. Hence, several recent studies have reported that lactoferrin might be able to minimize LOS and also NEC (49, 52). The ability of preterm infants to respond to pathogens, as the reported incidence of NEC and LOS indicates, inversely correlates to the gestational age (53), and enteral formula diets when coupled to parenteral nutrition predispose to NEC, while progressive nutrition with colostrum and mother's milk show a protective effect (50). Perhaps the increased amount of polyamines in breast milk known to have a protective effect on beneficial microbiota constitutes a natural contribution of mothers that give birth preterm (54, 55). Another interesting source of protection is related to the immaturity of the antioxidant defense system of preterm infants (55). Oxidative stress-derived free radicals are relevant contributing factors to a generalized inflammatory response, which sets the basis for organ/system damage (56, 57). Interestingly, it has been shown that feeding with preterm human milk is protective against hydroxyl radical aggression as compared to formula feeding. Hence, preterm babies fed own mother's milk eliminated significantly less biomarkers of oxidative damage to proteins and DNA in the urine as compared to paired formula fed preterm infants (58).

Fresh own mother's milk provided directly from the breast is the gold standard as all its biologically active components are preserved. Preterm infants require tube feeding and are either fed fresh expressed, frozen, and sometimes pasteurized donor human milk. Milk storage and processing affects bioactive compounds, but still donor pasteurized human milk appears to provide protection from NEC when compared to formula (59, 60).

ii) *Microbes to modulate preterm microbial composition*

Preterm infants are endowed with lower microbial diversity compared to term neonates. The use of probiotics could modulate towards a similar microbial community to those observed in term infant or adult gastrointestinal microbiome and ameliorate microbiome status of preterm infants. (61). Studies that promote exogenous supplementation with probiotics to preterm infants are based on the hypothesis that microbiota of preterm infants can be modulated by exogenous bacteria which results in an improvement in clinical outcomes. Probiotic bacteria are able to exert beneficial activity on intestinal epithelial cells, microbiota modulation and immune system response through different mechanisms of action which include: i) competitive exclusion, inhibition and displacement of potential pathogenic organisms adhesion and also, nutrient competition; ii) production of antimicrobial compounds; iii) improvement of barrier function; iv) modulation of immune response; and v) reduction of inflammation by interacting of NF-κB pathways (62).

The prevention of NEC in extremely low birth weight (ELBW) infants derived from the prophylactic use of probiotics has not yet been clearly established. In the most recent Cochrane review and meta-analysis on the prevention of NEC using prophylactic probiotics a total of 24 randomized and quasi-randomized studies were included. Prevention with probiotics, which contained either *Lactobacillus spp* alone or in

combination with *Bifidobacterium spp* was analyzed. Results of the meta-analysis suggested that probiotic supplementation may have some positive effects in relation with overall mortality and NEC, but did not influence the incidence of nosocomial sepsis. In addition, no side effects due to probiotic treatment were reported (48). However, it should be underscored that most of the studies included in this meta-analysis were not blinded, had a high variability in the enrolment criteria, in the feeding regimes, in the probiotic composition and dosing, and in the basal incidence of NEC and treatment regimes. Moreover, ELBW infants were underrepresented or not beneficial effects found in this specific population that represents the highest at-risk patients for NEC and/or septicemia. At present there insufficient evidence to recommend routine prophylactic use of probiotics to prevent NEC in ELBW infants. Moreover, there are still many unanswered questions regarding the use of probiotics in the clinical setting before its use gets generalized. Hence, identification of the appropriate probiotic-strain, rigorous quality control of manufacturing of this probiotic as a pharmaceutical agent rather than a food, dose, timing and length of treatment, effect on highest risk population or in exclusively breast fed infants, remain unanswered. Potential long-term consequences such as modification of host gene expression, influence on ongoing bacterial colonization, immune-modulation or antibiotic resistance have not been explored. Under these circumstances it has been recently proposed the need for a high quality NEC prevention clinical trial using probiotics in at-risk ELBW infants fulfilling the stringent guidelines of the International Conference of Harmonisation for Good Clinical Practice (63).

Interestingly, inactivated probiotics may also play a role modulating excessive inflammatory stimuli. Thus, preterm infants treated with inactivated probiotics showed a decreased incidence of NEC as compared to the control babies (64)

Further large clinical trials are required to answer these issues and to support and push the development of new probiotic products, which would benefit preterm population.

iii) *Prebiotics*

Prebiotics are non-digestible food ingredients such as oligosaccharides that are beneficial because they selectively stimulate the growth and/or biologic activities of intestinal bacteria in the colon thus contributing to ameliorate the host's health status. Prebiotics promote the growth of non-pathogenic organisms such as bifidobacteria. Only few studies have been performed in the clinical setting. The most recent updated review and meta-analysis which included a total of 7 studies and 417 preterm babies enrolled, concluded that the use of oligosaccharides enhanced beneficial bacterial growth but did not reduce the incidence of NEC, LOS, or modify time to achievement of full enteral feedings (64).

Conclusions

To conclude, perinatal and early postnatal time represent the most critical periods for the establishment of the microbiota, which exerts a key role in the establishment of the gut barrier and as an immune-modulator. Numerous perinatal factors influence neonatal microbiota such as mode of delivery, environment, hygienic measures, antibiotic use and breastfeeding practices. Preterm infants altered gut microbiota interaction with an immature immunologic intestinal response triggers pro-inflammatory and counter-inflammatory cytokine response. Breast milk is the gold standard for infant nutrition and influences the development of intestinal microbiota and immune system through its bioactive components. Probiotics may be promising in the prevention of NEC in certain populations of preterm infants. Future research should aim to explore appropriate treatment regimes, strain-specific effects on sub-selected populations and long term

effects of probiotic administration.

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Legend to Figure 1.

Part A, represents perinatal factors influencing gut microbiota composition in term and preterm infants (INTERVENTION TERM/PRETERM). Part B, shows the influences of perinatal factors upon microbiome composition in term and preterm infants. In preterm infants, antibiotic treatment, reduce utilization of human milk and prolonged hospitalization prompt the development of specific microbial strains that cause bacterial translocation, inflammation and oxidative stress thus contributing to the development of necrotizing enterocolitis (NEC) and/or late onset sepsis (LOS).

Table 1. Contributing causes to preterm delivery extracted from Muglia LJ & Katz M (reference #9)

Maternal conditions
- Preeclampsia
- Fetal distress

Preterm C-section

Reproductive assisted techniques
- Multiple gestations
- Older maternal age

Surgery performed in the mother

Social stress and race
- Low maternal age,
- Limited maternal education
- Poverty
- Unmarried status

Infection and inflammation
- Chorioamnionitis
- Inflammation of membranes

Genetic and epigenetic (environmental) factors

(A) INTERVENTION TERM/PRETERM

Breastmilk vs. Formula

Vaginal vs. C-section

Antibiotic treatment
Reduced utilization of human milk
Prolonged hospitalization

↑ Bacterial translocation
↑ Inflammation
↑ Oxidative stress

(B) MICROBIOME IMPACT

↑ *Bifidobacterium* spp

↓ Enterobacteriaceae

↑ *Lactobacillus*, *Prevotella* & *Sneathia* (maternal vaginal and intestinal microbiota)

↓ *Staphylococcus*, *Propionibacterium* and *Corynebacterium* (skin and oral microbiota, environmental bacteria)

Delayed Bacteroides colonization

↑ Proteobacteria and Firmicutes
↑ Enterobacteriaceae (E. coli & Klebsiella spp.)
↑ *Staphylococcus*, *Propionibacterium* & Corynebacterium

↑ NEC / LOS