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REVIEWS: CURRENT TOPICS

Nutritional modulation of the gut microbiota and immune system in preterm neonates susceptible to necrotizing enterocolitis☆

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Abstract

The gastrointestinal inflammatory disorder, necrotizing enterocolitis (NEC), is among the most serious diseases for preterm neonates. Nutritional, microbiological and immunological dysfunctions all play a role in disease progression but the relationship among these determinants is not understood. The preterm gut is very sensitive to enteral feeding which may either promote gut adaptation and health, or induce gut dysfunction, bacterial overgrowth and inflammation. Uncontrolled inflammatory reactions may be initiated by maldigestion and impaired mucosal protection, leading to bacterial overgrowth and excessive nutrient fermentation. Tumor necrosis factor alpha, toll-like receptors and heat-shock proteins are identified among the immunological components of the early mucosal dysfunction. It remains difficult, however, to distinguish the early initiators of NEC from the later consequences of the disease pathology. To elucidate the mechanisms and identify clinical interventions, animal models showing spontaneous NEC development after preterm birth coupled with different forms of feeding may help. In this review, we summarize the literature and some recent results from studies on preterm pigs on the nutritional, microbial and immunological interactions during the early feeding-induced mucosal dysfunction and later NEC development. We show that introduction of suboptimal enteral formula diets, coupled with parenteral nutrition, predispose to disease, while advancing amounts of mother's milk from birth (particularly colostrum) protects against disease. Hence, the transition from parenteral to enteral nutrition shortly after birth plays a pivotal role to secure gut growth, digestive maturation and an appropriate response to bacterial colonization in the sensitive gut of preterm neonates.

Keywords: Birth; Intestine; Milk; Bacteria; Inflammation; Development

1. Introduction

An increase in the number of induced vaginal deliveries and elective cesarean sections has resulted in an increased number of infants being born prematurely (<37 weeks). The tendency for earlier delivery is most evident in industrialized countries [1–3], partly resulting from advanced medical facilities which improve the survival rate of these preterm infants [4]. One of the consequences of preterm delivery is an increase in the proportion of preterm infants suffering from gastrointestinal complications, including necrotizing enterocolitis (NEC) [5]. NEC affects 1–7% [6] of the most preterm infants and mortality is very high.

Not surprisingly, an infant born prematurely shows various signs of organ immaturity and a corresponding inability to adapt appropriately to the various challenges of postnatal life. An organ that must immediately deal with microbiology, immunology and nutrition-related challenges is the gastrointestinal tract. As shown in Fig. 1, the immature gut is less able to deal with these challenges as a result of deficiencies in intestinal structural integrity, digestive capacity, intestinal immunity and dysregulated blood flow. Such deficiencies are some of the factors that are associated with increased NEC susceptibility in preterm versus term neonates. Identifying means of improving these deficiencies will aid in improving the maturation of the preterm gastrointestinal tract, reduce gut inflammation and improve outcomes for those predisposed to NEC.

NEC is found almost exclusively in infants fed a variable amount of enteral diet. The objective of this review is to discuss how the preterm gut responds to the first enteral food and how this response is associated with early bacterial colonization and the developing immune system. Better defining the optimal feeding regime remains an important aim because, despite variability in patient care among hospitals, the eventual goal is to transition all infants onto enteral feed. Based on our recent studies of the preterm newborn pig, combined with a review of the literature, we will examine how the diet and mode of feeding may influence gastrointestinal maturation, bacterial colonization and the sensitivity to inflammation. The strengths of animal models include a tight control of experimental conditions together with more insight into disease progression at the tissue and organ levels, compared with humans. On the other hand, it always remains a challenge to translate data generated from animal models to corresponding conditions in humans.

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2. NEC animal models

Several NEC animal models have been developed and studies in rodents, pigs and quails have been useful in advancing the understanding of NEC etiology [7–13], and they have overcome some of the limitations related to both infant studies and in vitro and ex vivo experiments. However, several aspects such as model species or model design must be considered before clinically relevant information can be extracted from animal studies. While some earlier reviews have focused on information derived mainly from rodent models [14], we give particular attention to the preterm pig. In contrast to rodent models, the size of the newborn pig easily allows for clinically relevant nutritional interventions (parenteral feeding) and the ontogeny and anatomy of the gastrointestinal tract is more similar to humans. The gut (and many other organs) in the newborn pig is more mature than in newborn rodents, although less mature

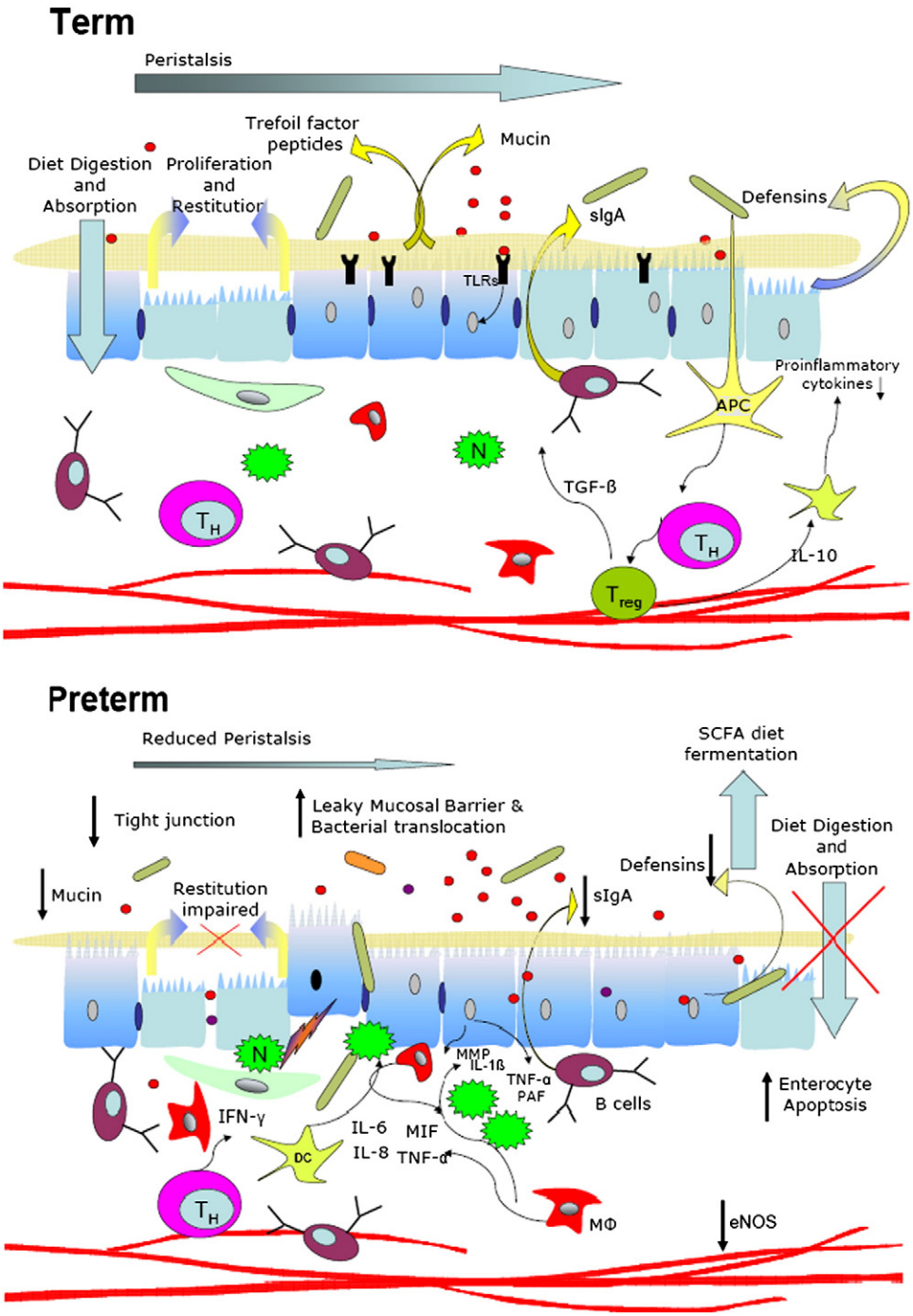

Fig. 1. Relative to term neonates (upper panel), preterm neonates (lower panel) show lowered digestive and nutrient absorptive function, impaired intestinal epithelial barrier and a dysregulated mucosal immune system. The immature intestinal barrier lacks several key protective mechanisms that normally prevent invasion by luminal bacteria. Maldigestion and a compromised gut barrier may render the mucosa susceptible to invasion by the gut microbiota, leading to the production of proinflammatory cytokines which further compromises intestinal defense mechanisms. The resulting imbalance between epithelial cell injury and repair leads to a vicious cycle of maldigestion, bacterial invasion, immune activation and uncontrolled inflammation. SCFA, short-chain fatty acids; N, neutrophils; Th, T helper cells; IL, interleukin; DC, dendritic cell; TGF, transforming growth factor; MIF, macrophage migration inhibitory factor; INF, interferon; TNF, tumor necrosis factor; sIgA, secretory IgA; eNOS, endothelial nitric oxide synthetase; MMP, matrix metalloproteinase.

than in infants [15]. Thus, delivery of pigs born at 90% gestation are comparable to preterm infants born at approximately 75% gestation (30 weeks), as evaluated from their degree of respiratory difficulties, feed intolerance, gut dysmotility, reduced digestive capacity and inappropriate immune response to luminal antigens [15]. NEC develops spontaneously in a proportion (5–10%) of preterm pigs and in very preterm humans (<30 weeks of gestation) in response to the initiation of enteral nutrition with mothers' milk [16,17], while incidences are notably higher after feeding artificial milk diets.

The clinical pathology of NEC in human infants is similar to that observed in preterm pigs. Abdominal distension, food intolerance, regurgitation, and lethargy are early clinical signs of NEC in both species [13,18]. Radiographically, 70–80% of NEC infants show signs of pneumatosis intestinalis, a hallmark sign of NEC, and this accumulation of gas produced by gas-forming bacteria in the submucosa, or subserosa, is also commonly observed in preterm pigs [13,16,19]. Histologically, NEC in both human infants and piglets causes pathological changes in all parts of the intestinal wall including, necrosis of enterocytes, edema, leucocyte infiltration and separation of the submucosal and lamina propria layers [20]. The histopathologic changes in the tissue reflect that of coagulative necrosis and can range from erosion of the epithelial layer to necrosis of the entire mucosa. Anatomically, the pathological changes are most often present in the distal small intestine and colon of both preterm pigs and infants; however, in severe cases, NEC lesions can be present throughout the gastrointestinal tract from the stomach to the rectum [21,22].

As the central paradigm for NEC development includes the interplay of nutritional, microbial and immunological determinants, it is important to have an animal model which allows independent studies of each of these determinants under clinically relevant conditions. In the following sections, we will describe how the preterm pig has been utilized to investigate the individual and combined role of these determinants of NEC development in preterm infants.

3. Nutrition

3.1. Enteral nutrition before birth and the role of amniotic fluid

While in utero, the human fetus swallows an estimated 100–200 ml of amniotic fluid per kilogram body weight per day [23], which may contribute as much as 10–15% of total energy and protein intake during mid to late gestation [24]. Amniotic fluid intake has a role in normal development of the fetal intestine and experimental fetal esophageal ligation results in both decreased body and small intestinal growth [24,25]. Interestingly, many of the beneficial factors present in amniotic fluid are also present in maternal milk [26,27], and such factors have putative functions in cellular growth and proliferation and cell-to-cell signaling [28]. For example, the presence of epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) indicate that amniotic fluid stimulates tissue and enterocyte growth in utero in addition to its effects on nutrient uptake postnatally [29,30]. Bioactive peptides and hormones in amniotic fluid also play an important role in fetal gastrointestinal development and immunoregulation [30,31], including potential anti-inflammatory effects of interleukin (IL) 10 [32] and transforming growth factor β (TGF-β) [33]. Further, it contains antimicrobial peptides such as defensins and lysozymes that provide protection against bacterial infection [34]. There is considerable functional and compositional overlap between the prenatal biology of amniotic fluid and the postnatal biology of mother's milk. In this manner, amniotic fluid intake in utero may function to prepare the gut for the dramatic shift from a highly controlled uterine environment to the challenging microbial and nutritional environment present immediately after birth.

It is possible that the bioactive properties of amniotic fluid in utero will also positively affect the immediate postnatal development of the intestine in preterm neonates. In preliminary experiments, we have determined the impact of porcine amniotic fluid on intestinal function in our preterm pig model of NEC [35]. Similar to porcine colostrum, postnatal administration of porcine amniotic fluid to preterm pigs reduced bacterial density and NEC severity, and induced differential regulation of many genes involved in gut inflammatory responses (see also Fig. 2, bar m). In comparing NEC pigs to colostrum-fed pigs, we confirmed tumor necrosis factor alpha (TNF-α) to be a pivotal regulator of several genes involved in both the inflammatory response and apoptosis, which is consistent with results in other NEC model studies [36–38]. We identified genes involved in inflammation [*CD55*, interferon γ (*IFN-γ*), *IL-1α*, *IL-2* receptor, *IL-4* receptor, *iNOS* (inducible nitric oxide synthase), TLR-3 (Toll-like receptor 3), TNF-α and TNF receptor associated factor] to be down regulated and genes associated with immunoregulation [TOLLIP (Toll-interacting protein) and IFN regulatory factor] to be up-regulated in pigs fed amniotic fluid when compared with NEC pigs. Together, the observations suggest that amniotic fluid may provide protection against intestinal lesions through suppression of inflammatory pathways, coupled with some antimicrobial activity.

3.2. Milk diets and gut development in preterm neonates

In preterm infants, relatively little is known about the trophic effects of enteral nutrition during the early neonatal period. The majority of information is extrapolated from animal model studies

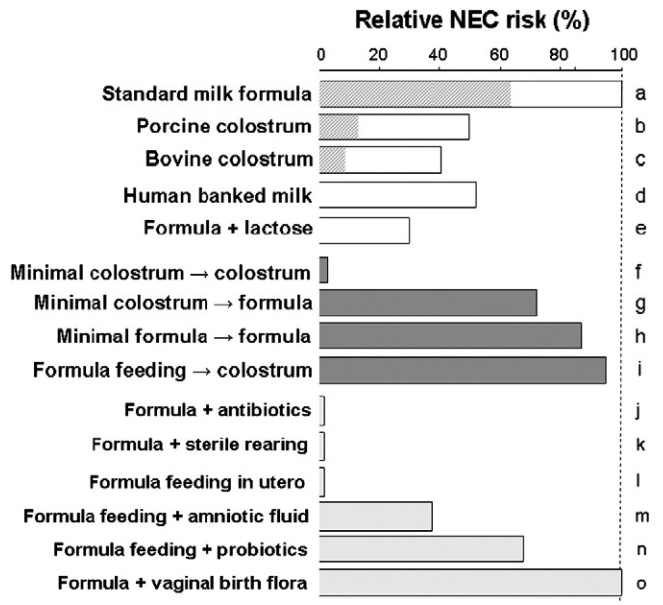

Fig. 2. Overview of preventive NEC interventions using the preterm pig model. NEC incidence following each intervention is expressed relative to that in control, 5 day-old, caesarean delivered preterm pigs fed total parenteral nutrition for 2–3 days followed by full enteral feeding with formula for 2 days (bar a, defined as 100% NEC sensitivity). Porcine colostrum, bovine colostrum and human milk all decrease NEC incidence (bars b–d), especially when enteral diets are fed immediately after birth without any parenteral nutrition (shaded bar parts in a–c). Lowered NEC risk is observed after addition of lactose to the standard polycose based formula (e) and feeding of small ("minimal") amounts of colostrum during the parenteral nutrition (f). Beneficial effects of minimal enteral nutrition are lacking using formula before and/or after the transition to full feeding (g–i). The limited or absent gut microbial colonization after antibiotics treatment (j), sterile rearing (k) or in utero feeding (l) completely prevents NEC. Finally, amniotic fluid (m) and probiotics (n) may reduce NEC via beneficial effects on the gut microbiota and immune system, while colonization of the preterm gut with the maternal microbiota after vaginal birth has no effect (o). See text for further details about the experiments.

where responses are generally believed to be similar to those for human infants [15]. There is mounting evidence that mother's milk is superior to infant formula in stimulating gut maturation, especially in the preterm infant where the digestive and absorptive functions are immature (Fig. 1). The presence of biologically active "milk-borne" growth factors present in mother's milk, but absent in formula, are believed to be the primary mediators of the effects of mother' s milk in the preterm gut. Such factors are largely resistant to proteolytic degradation and, thus, are able to exert a response in the targeted area of the gut by binding to specific receptors present on the mucosal epithelial cells [39]. Growth factors such as EGF, IGF-1 and TGF-β have all been shown to stimulate gut growth and maturation. Specifically, measures of villous height, epithelial barrier integrity, intestinal permeability, brush border disaccharidase and aminopeptidase activity and glucose transport are a few of the functional parameters shown to be improved in different animal species when fed specific growth factors present in mother's milk [40–42]. It should be noted that some studies have not found a growth difference between neonates fed formula or mother's milk, suggesting that macronutrients contained in the diet may be just as important as growth factors for gut growth [43,44]. However, these studies were performed in term neonates, supporting the view that the trophic response of mother's milk relative to formula may be most pronounced in preterm infants.

Equally important are the immunoprotective and immunostimulatory properties of milk on preterm gut development. Mother's milk provides both specific and nonspecific immunological factors that can enhance host defenses during the critical neonatal period. The important protective effects of mother's milk during the neonatal period is underscored by the observations of elevated levels of infections and gastrointestinal disease in preterm infants fed infant formula [13,17,45]. Specific immunofactors such as immunoglobulins (Ig; IgA, IgG, IgM, IgE, IgD), lymphocytes and phagocytic neutrophils and macrophages are present in milk and confer a protective defense against potential harmful bacterial antigens commonly encountered by the newborn [46]. Many of these factors are also involved in the stimulation of the neonatal immune system [47,48] (Fig. 1) and aid in the development of tolerance pathways critical for maintaining a balanced immune response to both noxious and harmless antigens. Tolerance is an active process, and ex vivo studies suggest that the dietary antigens present in breast milk, coupled with the immunosuppressive cytokines, aid in promoting tolerance to dietary and bacterial antigens [49]. Failure to regulate tolerance and active immune responses is hypothesized to contribute to food-related allergy, autoimmunity and inflammatory bowel disorders [50–52]. In addition, nonspecific factors such as lactoferrin, lysozyme, oligosaccharides, glycoconjugates and mucins are also present in mother's milk and have a general inhibitory effect, protecting against bacterial attachment, overgrowth and translocation [46]. All these factors, which are absent in formula, provide superior protective effects and immune system maturation in the developing gastrointestinal tracts of neonates.

Some of the diet-related differences in NEC sensitivity in preterm pigs are illustrated in Fig. 2. Porcine colostrum reduces the NEC incidence to less than half that of formula feeding (bars a+b Fig. 2) [13,17]. In preterm pigs, this protection by colostrum does not depend on whether the source is porcine or bovine (c, Fig. 2, Ref. [53]), or even human milk (d, Fig. 2, unpublished observations) suggesting that the colostral effects are partly species-independent. It is important however, that colostrum is provided throughout the neonatal period (bar f, Fig. 2). Only few hours of formula feeding is sufficient to initiate harmful inflammatory reactions in preterm pigs [54] which cannot be suppressed by subsequent provision of colostrum (i, Fig. 2, Ref. [55]). Interestingly, minimal enteral feeding with either colostrum or formula during the total parenteral nutrition (TPN) period is unable to prevent the detrimental effects of formula feeding during the subsequent phase of full enteral feeding (bars g–i, Fig. 2, Refs. [56,57]).

It remains a very important task to identify the NEC-protective factors that are lacking in infant formulas, relative to colostrum and mature milk. In addition to all the bioactive components referred to above, manipulation of macronutrients in enteral diets also warrants attention. For example, lactose-free formulas are often used as a precaution against infant lactose intolerance. Regardless, intestinal structure, absorptive function and NEC resistance are all markedly improved with a lactose- versus maltodextrin-containing diet in preterm pigs (e, Fig. 2, [58]). Although the optimal nutrient requirements may be partly species-specific, much work remains in optimizing the carbohydrate, protein, fat, mineral and vitamin levels of infant formulas to provide both sufficient body growth and development, as well as adequate NEC protection.

3.3. Parenteral nutrition and minimal enteral feeding

As shown above, enteral nutrition is an important and potent stimulator of gut growth during the neonatal period [59]. In the days after birth, the preterm infant may, however, be subjected to either TPN or parenteral nutrition combined with small amounts of enteral nutrition, often referred to as "minimal enteral nutrition." The clinical rationale for this intervention is to achieve a reasonable nutrient intake despite intolerance to enteral food and to avoid gastrointestinal overload until a degree of metabolic and hemodynamic stability has been achieved. Nevertheless, gut growth, villous height, mucosal mass, protein mass, cell proliferation and mucosal immunity are all indices significantly reduced by TPN feeding [60–64]. These deficiencies in mucosal structure and immune response are often implicated in development of increased intestinal permeability, bacterial translocation, and sepsis leading to increased neonatal morbidity or mortality [65]. Therefore, it is not surprising that some days of TPN will increase the susceptibility to NEC when enteral feeding, with either formula or colostrum, is initiated in the preterm NEC model (shaded versus nonshaded a–c bars in Fig. 2, Refs. [53,66]).

To date, a period of TPN in preterm infants has not conclusively been linked with increased NEC risk but TPN has been associated with other complications such as liver steatosis, cholestasis, thrombosis, and an increased susceptibility to inflammatory stimuli [65,67]. Furthermore, levels of gastrointestinal hormones such as glucagon-like peptide 2, peptide YY, insulin-like growth factor 1, gastrin, motilin, glucose-dependent insulinotropic polypeptide and vasoactive intestinal peptide are reduced in patients on TPN [61,68–70]. This can lead to the development of intestinal stasis commonly observed in preterm infants and can further contribute to bacterial overgrowth, bacterial translocation and sepsis [68]. Studies in piglets have shown that an enteral intake of 20% of the total nutrient requirement is needed to prevent gut protein loss [61], whereas normal gut growth and maintenance can be sustained with 40–60% intake [62]. Data from our group suggests that if enteral feeding is just ~10% of normal body nutrient requirements this will improve intestinal function (digestive enzyme activities, mucosal architecture and glucose absorption) and NEC resistance when bovine colostrum is used as the enteral diet but with limited effects when feeding infant formula [56]. Further studies are required to optimize the volume and nature of enteral diet required to induce maximal gut maturation and NEC resistance after preterm birth. However, observations such as these provide supportive evidence for the provision of small volumes of an optimal enteral diet during a parenteral nutrition period. This type of feeding regime has been shown to improve gastrointestinal motility and function [71] and may be an instrument to advance the establishment of full enteral feeding in preterm infants, an important goal of preterm infant care [72].

4. The gut microbiota

4.1. Microbiological colonization of the gut following birth

The transition from the mother's womb into the ex uterine environment is the most dramatic change experienced during mammalian life. Before birth, the fetal intestine is completely sterile [73]. Already during delivery, bacteria begin to colonize the gut and immediately start reshaping the luminal environment within their host [74]. These pioneer bacteria have been shown to modulate host-gene expression and function of the host epithelial cells [13,75] and can further influence the composition of the bacterial community during subsequent colonization [76]. In a healthy term neonate, adaptation to a new microbial condition is well tolerated due to sufficient development and maturation in utero. However, preterm birth places the newborn infant in a vulnerable situation, as adaptation to these conditions is compromised due to the underdeveloped gut (Fig. 1). Reduced intestinal motility, inappropriate immune response, decreased protective gastrointestinal secretions, reduced digestive and absorptive function and increased intestinal epithelial permeability are all conditions of prematurity that contribute to the vulnerability of preterm infants during this adaptation period [15,77,78]. Many of these deficiencies in the preterm gut increase the risk of intestinal dysfunction and diseases in response to bacterial colonization (Fig. 1). Although it is generally believed that NEC is not caused by the presence or absence of a single, or a few specific bacterial species, it is important to identify factors that contribute to the establishment of the first gut bacterial communities, as these are required for disease outbreak.

The use of germ-free animal models has been valuable for investigating the impact of bacterial colonization on early gut development. These models have shown that NEC does not occur in the absence of bacteria, whether following formula-feeding under germ-free conditions postnatally or following formula-feeding in utero (bars k and l, Fig. 2; Refs. [13,17,79]) The role of the microbiota is also supported by the marked decrease in NEC following the administration of broad-spectrum antibiotics to preterm pigs (bar j, Fig. 2 [95]). The colonization-dependent development of NEC may be accelerated by the supply of nutrients which are indigestible to the host but provide fermentable substrate for the microbiota. Specifically in our preterm pig studies, we have shown that carbohydrate maldigestion predisposes to NEC development, and NEC has consistently been associated with harmfully elevated concentrations of short-chain fatty acids and excessive luminal gas production causing abdominal distention [53]. Studies in germ-free preterm pigs also showed that early bacterial colonization affected not only the growth of gastrointestinal tissues (stomach, intestine, pancreas) but also the growth of other internal organs (e.g., spleen). This implies that variable hygienic conditions and the commonly used broad spectrum antibiotic administration to preterm infants may influence not only the gut but also affect organ growth in general [80].

Birth has profound influence on early colonization patterns, and differences in bacterial colonization between vaginal and cesarean delivery have been described [81–83]. In general, vaginally delivered infants are quickly colonized by fecal and vaginal bacteria of the mother [83,84] while infants born by caesarean section slowly acquire their pioneer bacteria from the environment [84]. Compared with term infants, the establishment of obligate anaerobes is significantly delayed in preterm infants [85–88]. This delayed colonization is more pronounced in cesarean versus vaginally delivered preterm infants, and results in persistence of facultative anaerobes including enterobacteria (i.e., *Escherichia coli* and *Klebsiella* species), enterococci (i.e., *Enterococcus faecalis* and *E. faecium*) and staphylococci (i.e., *Staphylococcus epidermidis* and *S. aureus*) [85–87,89]. A delay in the stable establishment of a normal bacterial community leaves the preterm infant more susceptible to bacterial disturbances. These disturbances in the bacterial community have been associated with an increased risk of gastrointestinal disorders and diseases [82,90–92]. Furthermore, it has been shown that the fecal microbiota of preterm infants is less diverse compared to term infants, consisting of only two to five species [73,85], and diversity is even further reduced in preterm infants born by cesarean section [93]. Similar to the negative effects of delayed colonization, decreased bacterial diversity may also predispose the preterm gut to bacterial overgrowth by potentially pathogenic species (e.g., *Klebsiella*, *Clostridium*, *Bacteriodes* and *Staphylococci* species), which may contribute to diseases such as NEC [12,13,15,94]. We have confirmed in pigs that bacterial colonization differs markedly between preterm and term pigs reared in the same environment [95], supporting the notion that maturity of the intestine (preterm versus term) have marked effects on the first microbial community in the gut.

Several studies in preterm infants have reported limited influence of delivery mode on initial colonization [85,87,96], while others have noted some differences [82,86]. Likely, the conflicting data regarding mode of delivery and early colonization reflects the difficulty in controlling for variables such as antibiotic treatment; length of hospital stay; intubation and the time, type and amount of enteral feeding [65,97–100]. All such conditions may affect initial bacterial colonization patterns and thereby minimize any influence of mode of delivery on preterm colonization. Although some attempts have been made to control for some of these variations, a more detailed characterization of bacterial colonization along the entire gut in preterm neonates is lacking, thus requiring the use of relevant animal models to study the main determinants of colonization and the associated changes on host tissues. In preterm pigs, we have demonstrated that newborn vaginally delivered pigs showed improved intestinal digestive and absorptive functions compared with caesarean-delivered pigs. In addition, we found that caesarean-delivered pigs had decreased bacterial diversity and density, and across delivery modes, formula-fed pigs showed significantly higher colonization by Clostridium species and higher NEC incidence [101]. However, NEC incidence was not significantly affected by delivery mode itself (see also Fig. 2, bar o). Thus, we show that enteral diet seems more important for the sensitivity to intestinal lesions than mode of delivery, although vaginal birth initially may induce more rapid gut maturation and a stable bacterial colonization. Using the preterm pig model of NEC, we have shown that the short term effects of diet are important for modulating the host response to bacteria, rather then significantly modifying the bacterial composition. As discussed below, this highlights the pivotal role nutrition plays in orchestrating the delicate balance between the gut microbiota and gut immunity to promote either gut maturation and health or gut disease (Fig. 3).

4.2. Dietary modulation of the gut microbiota

Comparisons of breast-fed and formula-fed infants indicates that diet influences the composition of the early bacterial community in preterm infants [89], although diet is often not the only variable that differ between such groups of infants. Normally, the differences are not observed until 1 week post-partum, despite the initiation of enteral feeding immediately after birth [102]. This is consistent with the observation that bacterial load and diversity does not show major diet-related differences in preterm pigs over the first 1–2 days of enteral tube-feeding [13,53,103]. In breast-fed infants, there is an increase in the relative abundance of bifidobacteria, and by the end of the first week, bifidobacteria are estimated to exceed the number of enterobacteria by a 1000-fold [104]. In contrast, enterobacteria levels in formula fed infants remain relatively high throughout the first week, while bifidobacteria are very slow to establish and are

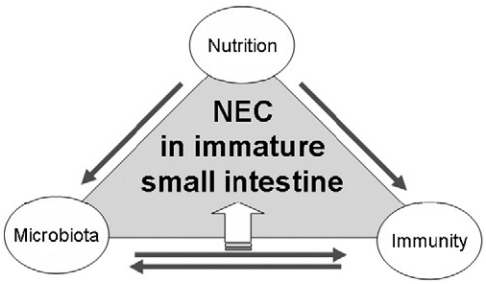

Fig. 3. Inflammatory lesions of the intestine in preterm neonates is to a large degree determined by three factors, nutrition, microbiota and immunity. The interactions between the gut microbiota and the immature immune system determine whether appropriate adaptation occurs. We conclude from studies in preterm pigs that nutrition plays a pivotal role in this interaction, and that a major part of the preventive measures against NEC should focus on nutritional interventions that improve the interactions between the gut microbiota and immune system. Using the preterm pig, feeding increasing amounts of colostrum already from birth is effective in protecting against bacterial invasion and overgrowth, as well as in facilitating a well-controlled immune response.

only about 10% of the level seen in breast fed infants [104]. This delayed colonization by bifidobacteria in formula-fed infants has stimulated research into identifying a specific "bifidus factor" or other dietary components present in breast milk (e.g., prebiotic oligosaccharides), that would promote the growth of both *Lactobacillus* and *Bifidobacterium* species when added to formula [105–109]. Identifying such factors is particularly important for preterm infants, because growth of these beneficial bacteria is delayed in preterm infants, especially in those fed formula [85,110,111]. Stimulation of these beneficial bacteria in preterm infants may act to suppress the growth of potential pathogens known to proliferate in the less colonized preterm gut of formula fed infants and cause disease [12,17,112,113]. As previously mentioned, bacterial diversity is reduced following preterm delivery, but compared with formula feeding, bacterial diversity has been shown to increase with breast feeding [85]. This may act to protect the preterm gut from the proliferation of single pathogenic species, a characteristic feature of critically ill infants [113]. In preterm pig studies, we have shown that the differences in bacterial population depends more on the disease status (NEC or healthy) than on the type of enteral food (colostrum versus formula), and that *Clostridium perfringens* is frequently abundant in pigs diagnosed with NEC [53,103]. On the other hand, inoculation with clostridia has not consistently increased NEC incidence [95]. Together, the results from preterm pigs suggest that low bacterial diversity and early feeding-induced overgrowth with specific species are more a result of the disease process than the major cause of NEC [114].

TPN feeding may compromise the integrity of the gut in the neonate [115,116], delay bacterial colonization, reduce bacterial diversity and thereby selectively promote the proliferation of potential pathogens such as *C. perfringens* [112,117]. This is consistent with our observation that TPN increases the risk of NEC in preterm piglets. We have also shown that Gram-negative bacteria dominate the preterm pig intestine after 48 hours of TPN [53], thus increasing the risk for bacterial translocation [118]. Mucosal colonization density increases immediately following the transition to enteral feeding, particularly using formula (relative to colostrum), facilitating a pronounced Gram-negative proinflammatory (IL-1β) state of the preterm intestine. This initial proinflammatory insult after TPN may then subsequently affect regulators of small intestinal motility and tissue perfusion as indicated by the formula-induced adaptational increases in glial cell density, as well as neuronal nitric oxide synthetase (nNOS)- and vasoactive intestinal peptide (VIP)-containing neurons [119,120]. Although the feeding-induced decrease in endothelial nitric oxide synthetase, reflecting maladaptation of the intestinal microvasculature, is relatively diet-independent, only formula-fed pigs show increased hypoxia sensitivity, as indicated by hypoxia-inducible factor-1 alpha (HIF-1α) expression [121] (Fig. 1).

The increased interest in probiotic administration during the early neonatal period is due to observations that probiotics can improve gut health and function leading to a decrease in disease. Both clinical trials and animal model studies have strengthened the understanding of how probiotics function within the gut of the host and have shown that probiotics can positively influence innate immune response to antigens, digestive and absorptive capacities, epithelial barrier integrity and tight junction formation; alter the microbiota and increase resistance to pathogen colonization [12,122–126]. Much of the research to date has focused on the administration of relatively few strains of *Bifidobacterium*, *Lactobacillus* and *Streptococcus*, emphasizing the need for more studies investigating the therapeutic potential of unexplored probiotic candidates.

The administration of probiotics to preterm infants is of particular interest because the establishment of a "normal" microbiota is often impaired and the acquisition of bifidobacteria and lactobacilli is delayed. In human clinical trials, administration of bifidobacteria and lactobacilli bacteria to preterm infants have resulted in a reduction in the incidence and severity of NEC [125,127,128]. The practical and ethical limitations in human clinical studies have prevented an in-depth investigation into the potential action of probiotics in infants. In rodent and quail animal models, probiotic bacteria have been shown to inhibit the colonization of potential pathogens such as *C. perfringens*, decrease the expression of proinflammatory mediators and reduce endotoxin translocation, thereby reducing the incidence and severity of NEC [11,12]. These studies provide supportive evidence for the potential application of probiotics to preterm infants, and continued use of relevant animal models will be important for characterizing the underlying modes of action responsible for these improved responses. In our own studies [103], boluses of probiotics (*Bifidobacterium animalis* and four *Lactobacillus* species were administered to preterm pigs immediately after birth by caesarean section to provide the probiotics with a competitive advantage in becoming the predominant bacteria to initially colonize the newborn gut. We demonstrated that probiotics reduced the diet-induced mucosal atrophy, dysfunction and NEC severity (see also Fig. 3, bar n), while improving the growth of resident non-probiotic lactobacilli and decreasing the density of the potential pathogen, *C. perfringens* [103]. Regardless, the optimal timing, number and identity of species, as well as the amount and frequency of the supplementation, are largely unexplored and may be important issues particularly in the neonate where the intestinal microbiota is fluctuating and unstable. In fact, in a later study, we showed that early neonatal probiotic supplementation with a pool of either viable or killed probiotic strains had negative effects on NEC resistance in preterm pigs [129]. Hence, the clinical paradigm for routine probiotic supplementation to premature neonates, should be established with great caution.

5. Gut immunity

5.1. Mucosal immune and bacterial defense systems

Gut bacterial colonization plays a key role in development of the local gut immune system as well as immune development in other organs and in the circulation to enable the host to protect itself against pathogenic microorganisms. The first line of defense against invaders consists of physical and chemical barriers: mucous membranes that line the respiratory and gastrointestinal tracts and chemical barriers containing a series of enzymes and other substances that have a direct antimicrobial action or inhibit microbial adherence to body surfaces (Fig. 1). Microorganisms that cross this first line of

defense will activate components of the innate immune system and subsequently, the adaptive immune system. Innate immunity involves elements such as complement system proteins, acute phase proteins, cytokines and cellular elements such as monocytes, macrophages, granulocytes, dendritic cells and natural killer lymphocytes. The innate response has limited capacity to distinguish between microorganisms and often has a similar response to many different microorganisms [130].

Toll-like receptors (TLRs) are membrane spanning receptors that recognize pathogen-associated molecular patterns on microbes (PAMPs) once they have breached the physical barriers, such as the mucus-layer, and therefore play a key role in the innate immune system. To date, there are 13 identified TLRs that recognize an assortment of PAMPs [i.e., lipopolysaccharide (TLR-4), peptidoglycan (TLR-2), flagellin (TLR-5) and bacterial DNA (TLR-9)]. All currently recognized TLRs are homologous with the IL-1 receptor, sharing an intracellular signaling domain, known as the Toll/IL-1R domain [131]. For example, the interaction of TLR-4 with lipolysaccharide leads to the activation of myeloid differentiation primary response protein 88-dependent signaling, resulting in the induction of pro-inflammatory genes such as TNF-α, IL-1β and IL-6 [132] (Fig. 1). It has also been shown that TLR-4 signaling is involved in intestinal inflammation. High TLR-4 expression has been associated with human NEC [132] and with NEC in experimental animal models [133–135]. Similarly, we have also shown that TLRs (2 and 4) are up-regulated in intestinal tissue from preterm pigs with NEC. Hence, TLRs play a pivotal role in mucosal inflammation and under basal conditions. Epithelial-bacterial interactions that may occur via TLRs are likely to play roles in the regulation of processes that regulate barrier integrity, such as epithelial migration, proliferation, and apoptosis. During states of stress, such as hypoxia, TLR signaling becomes exaggerated in response to ligands, thus favouring mucosal barrier disruption and adversely affecting mucosal repair while worsening mucosal injury [131]. In recent proteomics studies on preterm pig intestinal tissue, we showed that heat shock proteins (e.g., HSP60 and GRP78) were increased after just 8 h of formula feeding [79,136,137], suggesting that stress proteins released in response to formula feeding may initiate the inflammatory cascade through TLRs. Comparing the differences in these inflammatory signaling pathways between preterm and term infants, the latter being more capable of tolerating bacterial colonization (Fig. 1), may be critical to identify therapies to prevent inflammatory diseases in preterm neonates.

Immune system development begins early in the embryonic period but continues to mature up to about one year of age [138]. This results in an "immunocompromised" neonate at birth. The immature mechanical barriers, the limited functions of neutrophils, the low plasma concentration of specific antibodies, the low activity of complement system proteins and the poor cooperation between T and B lymphocytes predispose these infants to disease (summarized in Table 1). These impairments are directly related to degree of prematurity. Thus neonates, especially preterm neonates, are prone to bacterial infection and inflammatory diseases with substantial morbidity and mortality [140,147] (Fig. 1). This further highlights the importance of early nutrition in preterm neonatal well being and survival. Most available data regarding ontogenesis of the vertebrate immune system are derived from human and rodent studies. Relatively limited data are available in other species but those that exist suggest similar patterns of immune development [156]. Still, the stage of development achieved at the time of birth and, thus, the degree of immunocompetence in early postnatal life vary from species to species. In addition, species vary widely in the degree to which passive immunity, in the form of colostral immunoglobulins, is taken up by the neonatal intestine but this capacity is also reduced by preterm birth [17]. Therefore, degree of prematurity is a key factor when considering animal models with similar immune development.

Table 1
A summary of some immune system characteristics in preterm neonates

Component	Description in preterm neonates	Reference(s)
First line defense		
Mechanical barriers	↓ Stratum corneum function	[139]
Intestinal mucosa	↓ IgA, IgM, IgG	[140]
	↑ Intestinal permeability	[141]
	↓ Antibacterial peptides	[142]
Innate immune response		
Antigen presenting cells	↓ MHC receptors	[143,144]
Complement system	↓ Protein level and activity	[145]
Neutrophils	↓ Number	[146]
	↓ Adhesion	[147,148]
	↓ Oxidative burst	[146,149]
	↓ Deformability	[150]
	↓ Phagocytosis	[147]
Natural killer cells	↓ Number and function	[151]
Adaptive immune response		
Lymphocytes	↓ T and B cell count	[152]
	↓ T helper	[140]
	↓ Cytotoxic lymphocytes	[151]
	↑ T regulatory (inhibitory) cells	[153,154]
	T-helper 2 polarized	[155]

Maintaining epithelial barrier integrity is critical to intestinal homeostasis. A breakdown in this protective barrier function can quickly lead to the translocation of luminal bacteria, resulting in an exaggerated immune response (Fig. 1). The idea of intestinal barrier compromise leading to intestinal inflammation has been considered as a possible condition leading to NEC. This would suggest that improving the intestinal barrier integrity in the susceptible preterm infants may be critical in preventing the deleterious inflammatory response. Inadequate intestinal restitution results in breakdown of intestinal integrity and this has been suggested to be a critical step towards the development of NEC in rodents [157]. Other studies have investigated the therapeutic potential of administering factors known to bolster epithelial barrier integrity (i.e., EGF, glucagon-like peptide 2, trefoil factors [7,13,158,159] and some have shown a reduction in disease, although not complete prevention. Further investigation into how the initial epithelial barrier is compromised may lead to new therapeutic factors capable of preventing the initial breakdown of epithelial barrier integrity.

5.2. Dietary modulation of mucosal defense and immunology

As previously stated, preterm neonates have several factors that favor inflammatory disease pathology potentially leading to irreversible tissue damage. The mechanisms whereby mother's milk and colostrum protects against NEC may be similar to those induced by providing anti-inflammatory components; growth factors; erythropoietin; lysozyme; immunoglobulins; antibacterial peptides as well as pre- and probiotics, amino acids and nucleotides [116,160–165]. Using preterm pigs, we have shown that colostrum increases the Goblet cell mucous production as a first line defense against bacterial attachment and invasion [166]. Specific dietary components may also be effective and the potential to modulate the activity of the immune system by interventions with specific nutrients is termed "immunonutrition." Some components have already been extensively studied (e.g., arginine, glutamine, branched chain amino acids, polyunsaturated fatty acids) at several target sites (mucosal barrier function, cellular defense, local or systemic inflammation responses) [167].

Arginine is involved as a substrate for generation of nitric oxide, a vasodilator that is involved in intestinal permeability, mucosal integrity and barrier function [168]. Plasma levels of the amino acids arginine and glutamine are significantly lower during the early neonatal period in preterm infants who develop NEC compared with similar gestation infants who do not develop NEC [169] and arginine

supplementation reduces NEC [170]. However, it is unclear whether the apparent beneficial effects of arginine are related to subsequent synthesis of glutamine [171] or the free radical scavenging properties of arginine itself [171,172]. Glutamine is the preferred fuel for rapidly proliferating cells including enterocytes and is abundant in mother's milk but present in much lower levels in formula milk; thus, glutamine supplementation may enhance mucosal integrity and intestinal barrier function in preterm neonates.

Long-chain polyunsaturated fatty acids (PUFAs) have been proposed to modulate inflammation and immunity [173] and preterm neonates fed formula supplemented with PUFAs (i.e., phospholipids) showed reduced NEC [174]. It has been shown that PUFAs can specifically down-regulate platelet activating factor receptor gene expression in the ileum and colon of rats, suggesting a possible mechanism for the protective effect on the intestine [175]. Platelet activating factor (PAF), an endogenous phospholipid with powerful proinflammatory properties, is synthesized in response to exposure to lipolysaccharide and hypoxia by multiple cell types, including enterocytes, and has been implicated in intestinal necrosis [9,36,37,176,177]. Human neonates with NEC show high levels of PAF and decreased PAF-acetyl hydrolase (degrades PAF) with levels correlating to NEC severity [178]. The presence of PAF acetyl hydrolase in mother's milk [179,180] may, in part, explain the protective effects compared with formula.

Growth factors such as EGF, IGF-1, TGF-β and hepatocyte growth factor have trophic effects on the fetal and neonatal gastrointestinal tract [181], indicating that growth factor supplementation may facilitate intestinal barrier maturation and prevent the host from bacterial translocation. Both amniotic fluid and mother's milk contain the aforementioned growth factors [27], indicating their importance in the transition to the ex uterine environment. Preterm neonates, and neonates suffering from NEC, have reduced levels of saliva and plasma EGF [182] while EGF supplementation appears to increase NEC resistance [7]. In a hypoxic rodent NEC model, EGF down-regulated proinflammatory cytokines and up-regulated anti-inflammatory cytokines [183], supported maintenance of intestinal barrier by goblet cell proliferation [184] and inhibited intestinal cell apoptosis [184,185].

6. Conclusion: nutritional modulation of microbiota-immunity interactions in NEC

The available literature, combined with our own results on preterm pigs (Fig. 2), show that the type of enteral diet (mother's milk, formula, immunonutrients, amniotic fluid, probiotics) and mode of feeding (parenteral, enteral or minimal enteral) play significant roles for neonatal gastrointestinal development and NEC resistance. Infant formulas fail to replicate the important maturational and protective effects of mother's milk which may be explained by the lack of bioactive constituents as well as an inappropriate nutrient composition. Mother's milk promotes intestinal growth, immune modulation and maintenance of a healthy microbial environment. This is particularly important for preterm neonates since deficiencies in intestinal integrity, barrier function, digestive capacities and intestinal immunity (Fig. 1) lead to increased susceptibility to inflammatory diseases. We believe that enteral nutrition has great potential to manipulate the local actions of the gut microbiota, in parallel with direct effects on the mucosal immune system, to prevent the onset of NEC (Fig. 3). Bacterial colonization is a crucial determinant of NEC and may be modified via diet although the main benefit of an optimal diet in preterms appears to be prevention of the harmful mucosal actions of the resident gut microbiota rather than induction of a fundamental change in the microbiota composition. It is possible that even a normal bacterial community has the potential to cause disease provided that the (diet-related) defense mechanisms are suboptimal. On the other hand, administration of probiotics has been shown to improve gastrointestinal function and reduce NEC outbreak, indicating that specific groups of bacteria indeed act to improve the integrity and function of the gastrointestinal tract. It remains however, that the beneficial effect of probiotics may occur mainly by providing a temporary competitive advantage for certain nonpathogenic resident bacteria that may reduce the potential damaging effect of specific pathogens.

Identifying and understanding the early events in the etiology of NEC will be crucial for improved preventive inventions and clinical therapies for this devastating disease. Using preterm pigs, we have shown that feeding infant formula, instead of mother's milk, as the initial enteral diet to preterm neonates rapidly results in mucosal inflammatory responses, even after only a few hours of feeding [136,186]. These rapid adverse effects may be impossible to reverse with later nutritional, antimicrobial or pharmacological interventions. Therefore, we emphasize the need to clearly understand the early preclinical events leading to disease in order to identify relevant early preventative interventions, particularly when mother's milk is not available. To achieve this goal, we need a continuous refinement of clinically relevant animal models as well as coordinated efforts to test promising new treatments for this vulnerable patient group.

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