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Breast milk: A modulator of the immature immune system in the management of necrotising enterocolitis in preterm neonates

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ABSTRACT

Necrotising enterocolitis remains (NEC) a major source of morbidity and mortality among preterm infants. The use of breast milk is a major protective factor against NEC, with its anti-infective nutritional and immunological properties. Breast milk, expressed either from the mother's own milk, or in the form of pasteurised donor milk, is the preferred nutritional source of enteral feeding for very low and extremely birth weight infants. Although there is a lack of definite data, breast milk is superior to preterm commercial formulas. However, breast milk seems not only to protect the immature bowel of preterm neonates but also treat the immature host defense system of the gut. The present review presents the currently available data in the literature on the diverse aspects of the role of breast milk not only as a useful feeding strategy to prevent NEC, but also as a means to treat the immature gut of preterm infants.

Key Words: *Breast milk; intestinal microbiota; necrotising enterocolitis; premature neonates; preterm formula*

INTRODUCTION

Necrotising enterocolitis (NEC) is the most common acquired neonatal disease of the gastrointestinal tract (GIT). The pathogenesis of NEC is not clearly elucidated and consequently questions arise on how to treat or prevent the disease [1]. NEC incidence may vary significantly between different neonatal intensive care units. The overall prevalence of NEC is estimated to be 1 to 3 per 1000 live births [2]. Ninety per cent of NEC affected neonates are premature, and 14% of them are weighing less than 1000g. Notably, 50% of the extremely low birth weight neonates may need a surgical intervention. Despite advancements in the treatment of NEC, the mortality rates in premature neonates remains high, ranging from 10% to 50% [1,2].

Advances in medical knowledge have demonstrated that breast milk (BM) is the most favourable source of nutri-

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tion of neonates and its health, nutritional, immunological, developmental, and physiological benefits are universally established [3]. In addition, numerous studies have shown that BM minimises the occurrence and severity of NEC in premature neonates by reducing the influence of risk factors in the development of NEC [4,5]. On the basis of these data, the present review aims to investigate the effectiveness of BM in the management of NEC. We sought to test the hypothesis that after reviewing the current literature, we could propose BM not only as a means to prevent NEC, but as a modulator of the immature gut of premature neonates.

METHODS

The review of the literature was performed through PubMed and Google Scholar databases. Inclusion criteria concerned original and review articles, systematic reviews and meta-analyses published from January 1, 1980 to December 31, 2023, without language restrictions. The search strategy was performed using the Boolean operators AND/OR. The search terms used were: human milk OR breast milk OR breast feeding OR enteral nutrition OR donor milk OR human-milk- based fortifiers AND premature neonates OR low birth weight neonates OR very low birth weight neonates OR extremely low birth weight neonates AND intestinal microbiota OR bioactive factors AND necrotizing enterocolitis OR experimental necrotizing enterocolitis OR intestinal inflammation. Furthermore, the references of the articles were investigated by hand for related articles. All articles were selected systematically for inclusion and critically evaluated.

Necrotizing enterocolitis

1. Factors associated with pathogenesis of NEC in premature neonates

Although it is generally accepted that NEC is a multifactorial disease, preterm birth, the gastrointestinal microbiota and the intestinal immaturity are the major risk factors for the pathogenesis of NEC in premature neonates. Intrauterine infection is the leading cause of preterm birth and recent research focuses on the association of uterine microbiome and preterm birth [6]. The role of gastrointestinal microbiota is very important in protecting mucosal integrity and alterations in its synthesis may lead to sepsis, NEC and systemic inflammatory bowel disease. Intestinal immaturity related factors such as impaired gut motility, digestion, absorption, barrier function, immune defense and circulatory regulation, may be responsible for the pathogenesis of NEC in premature neonates [7]. Furthermore, additional factors such as genetics, the type of diet, e.g. human milk versus formula, the exposure to antibiotics and the mode of delivery, all may contribute to NEC development [8-10].

2. Gastrointestinal microbiota: from fetal life to early postnatal days

2a. Fetal gastrointestinal microbiota

Although GIT is thought to be sterile in normal fetuses, with microbial colonisation of the gut beginning at birth from vaginal bacteria in vaginal delivery or from maternal skin surface and the surrounding milieu in caesarian section, cultured-dependent studies have discovered microorganisms in amniotic fluid, fetal membranes, umbilical cord, and placenta [11]. Moreover, several studies based on non-cultured dependent studies by using high throughput 16S ribosomal RNA gene analysis, revealed the presence of substantially diverse assemblages of bacteria, such as *Enterococci and Staphylococci*, isolated from human meconium, obviously suggesting that this material was composed during fetal life [12,13].

2b. Early postnatal life

The early postnatal life comprises a period of roughly 7-9 days, during the course of which the development of intestinal microbiota is settled [14]. In full-term neonates, the first bacteria that colonize the intestine include *Streptococcus*, *Staphylococcus*, *Escherichia coli*, *Lactobacillus*, and *Enterobacter* species. These bacteria by consuming oxygen produce a low oxygen environment, giving the opportunity to grow and, finally, prevail anaerobic bacteria species such as *Clostridia*, *Bifidobacterium* species and members of *Firmicutes* phyla [14]. However, other factors, including the mode of delivery and diet play a significant role in the initial colonization. More specifically, neonates born vaginally are seeded with maternal vaginal flora, such as *Lactobacillus* and *Prevotella* species, while those born by caesarian section are colonised by skin flora, such as *Staphylococcus* and *Corynebacterium* [15]. Moreover, neonates born by caesarian delivery show a decreased amount of *Bifidobacteria* and a delay in colonization [15]. BM is another potential provider of bacteria to the neonatal gut such as *Staphylococcus*, *Streptococcus, Lactobacillus* and *Weissella* species. Nevertheless, full term neonates fed with formula show a different microbial pattern, promoting the presence of *Enterobacteriacea*e, *Bacteroides* species, and *Clostridium difficile* [16]. Yet, there is a controversy concerning the amount of *Bifidobacteria* provided by formula fed neonates, which may reflect the different composition of formulas [15].

Preterm neonates are characterised by some unique characteristics, usually derived from an unexpected delivery due to an inflammation of the maternal/fetal membrane. For instance, preterm neonates are fed earlier and usually by formula milk, they are exposed to antibiotics and they are usually grown up in a hospital environment that is host to many atypical bacteria [16]. Consequently, the preterm intestinal microbiota is characterised by a decreased overall diversity and a different microbial load than those of full-term neonates, consisted by bacteria, such as *Escherichia coli*, *Staphylococcus* and *Klebsiella* species, facultative anaerobes, such as *Enterobacteriaceae*, *Enterococcaceae*, and *Weissella* and decreased proportion of beneficial bacteria such as *Bifiiobacteria* and *Lactobacillus* [17].

2c. The impact of the preterm intestinal microbiota in the pathogenesis of NEC

Intestinal dysbiosis with lower microbiota diversity has been found to be related with the development of NEC in preterm neonates. Mai et al. [17], by comparing the intestinal microbiota of premature neonates with NEC with this of unaffected control neonates, found an increase of 34% of *Proteobacteria* and a decrease of 32% of *Firmicutes,* in samples collected one week but less than 72 hours prior to NEC, but not in matched samples. Similarly, Torrazza et al. [11], by analysing fecal samples of premature neonates, using 16S rRNA methods, at two, one and zero weeks prior to NEC development, found a higher proportion of phylum *Proteobacteria* (61%) and *Actinobacteria* (3%) two weeks and one week respectively compared to controls, and lower numbers of *Bifidobacteria* and *Bacteroides*. Additionally, certain bacteria of the *Klebsiella* genus were found before the NEC presentation. A prospective control study evaluated the intestinal microbiota between premature neonates, who developed NEC, and unaffected controls. They revealed that in the early onset of NEC, the abundances of *Clostridium sensu stricto* were much higher than those of case controls, while in the late onset of NEC, Gammaproteobacteria (*Escherichia coli, Shigella*, and *Cronobacter*) predominated and were significantly higher than controls [18]. They suggested that the precise infectious agent of NEC may change by the age of premature neonates, while antibiotics administration may have an impact on the microbial diversity [18]. Moreover, in a control study, Heidi et al. [19] reported the existence and plethora of *Clostridium perfringens* and *Bacteroides dorei* in meconium samples of preterm neonates (aged 24-29 weeks) who developed NEC, compared to those who did not. They suggested that the pre-existence of a NEC-linked gut microbiota with *Clostridium perfringens* and *Bacteroides dorei* in the meconium, predisposes to a NEC-associated microbiota development. Conflicting are the findings of Wang et al. [20] and McMurtry et al. [21]. The former noticed low microbiota diversity in neonates with NEC, an increase in *Gammaproteobacteria* and decrease in other bacteria species, suggesting the impact of a single dominant microorganism responsible for NEC. The latter reported that bacterial diversity tended to decrease with the severity of NEC and lack of Clostridia in lethal cases of NEC, suggesting the perception of bacterial dysbiosis. In summary, it seems that the intestinal microbiota of preterm neonates, who develop NEC later, is different than those who do not. Most studies suggest that there is not a distinct pattern of intestinal colonisation associated with NEC development, while the age of onset may be an additional contributing factor to the microbial colonisation of the intestine.

3. The GIT host defense system

3a. Physical barriers

Epithelial cells represent the physical barrier of the intestinal lumen from the other parts of the human body. The integrity of this barrier is sustained by the presence of tight junction among epithelial cells comprised by enterocytes, goblet cells and Paneth cells. Enterocytes not only provide a physical barrier, but also produce a substantial number of immunomodulatory factors [22]. The recognition of bacteria is first made by Toll like receptors (TLRs) molecules expressed by enterocytes. Among different TLRs molecules, TLR4 is implicated in the pathogenesis of NEC [23]. Goblet cells, first recognised at 9 to 10 weeks of gestation, are involved in the secretion of mucin glycoproteins, which generate the mucus layer of the intestine. Mucus layer supports the underlying epithelium from digestive enzymes and bacterial toxins, and any loss in its production or composition may allow bacteria invasion and induction of NEC [24]. Paneth cells are specialised epithelial cells located in the crypts of Lieberkühn secreting defensins and other anti-microbial peptides that kill invasive pathogens, frame the intestinal microbiota, protect the intestinal stem cells from pathogens, trigger the stem cells differentiation, and participate to the regeneration of blood vessels after injuries to the gut.[24] According to the hypothesis of McElroy et al. [25], Paneth cells have a pivotal role in the onset of NEC in premature neonates. Destruction of Paneth cells by microbial toxins leads to bacterial invasion, severe inflammation, pneumatosis intestinalis and vascular closure in the submucosa, triggering the ultimate pathway to NEC. Tight junctions

between epithelial cells restrict the translocation of bacteria, while helping the absorption of macromolecules produced during the process of digestion [25]. Immaturity in the composition and function of tight junctions lead to increased permeability of the epithelial barrier to the products of bacteria such as lipopolysaccharide, which in turn stimulate the secretion of several pro-inflammatory cytokines by the epithelial cells, involving tumor necrosis factor (TNF), IL-6, IL-8, all of them contributing to the distinctive inflammatory process of NEC [25].

3b. The gut immune system-the role of enterocytes in NEC

Directly below the epithelial barrier, specific immune cells reside that are capable of initiating immune feedback. These include macrophages, dendritic cells, T cells and B cells. Furthermore, specialised epithelial cells, called microfold cells (M cells), are believed to act as an antigen sampling system. Any damage to M cells may lead to an increased uptake of microorganisms, as it may be seen during gut inflammation [26].

Activation of the immune system is achieved by the recognition of a pathogen-associated molecular pattern by the host immune pattern recognition receptors (PRRs). TLR4 is an important PRR that recognises lipopolysaccharide (LPS), a crucial endotoxin in the pathogenesis of NEC and its activation in the premature gut is required for the development of NEC [27]. Accordingly, strategies that inhibit TLR4 signaling, including amniotic fluid, breast milk, and genetic deletion of TLR4 from the intestinal epithelium in animal models, constrict the NEC severity [28,29].

The expression of CD14 (cluster of differentiation 14, the co-receptor of LPS) on enterocytes is thought to be an important factor in the induction of NEC [30]. Enterocytes are capable of producing large amount of interleukin-8 (IL-8) in response to LPS, bacteria or inflammatory cytokines, and compared to control neonates, premature infants have higher levels of IL-8 in NEC affected tissue and serum [31]. In conclusion, enterocytes are not exclusively a physical barrier but also participate in gut homeostasis by secreting and activating various immunomodulatory factors in response to various strains of NEC-associated bacteria.

BM

A substantial number of short- and long-term studies have documented BM as the normative standard for infant feeding and nutrition [31]. The beneficial properties of BM are based not only on nutrients, but also on various bioactive compounds with growth, anti-pathogenic and anti-inflammatory properties, that play a pivotal role in neonate health and survival [31] (Figure 1). BM is a potential native immune system that protects mother's offspring through three ways: a) inhibition of pathogen binding, b) prebiotic activity, and c) immune control, and adjustments of infection [32]. The impact of immunomodulating components of BM on NEC are shown in Table 1.

Immunomodulating components of BM and protection against NEC

Lactoferrin

Lactoferrin is an iron-binding glycoprotein that acts as a part of inherent immune system and is found in human BM [33]. It acts by many mechanisms which target in the protection of intestine from systemic infection and NEC in premature neonates: a) in stomach, under the influence of pepsin is transformed to lactoferricin which acts against gram-negative bacteria by disrupting their cell-membrane, b) synergistically with lysozyme may kill gram-negative bacteria in the stomach, c) it may bind to TLR4 and CD14 receptors blocking the adherence of bacteria to the intestinal epithelium, d) it promotes the apoptosis of infected intestinal epithelial cells, e) it stimulates the growth of commensal bacteria, and f) reduces the production of inflammatory cytokines, such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and IL-8 via blockage of nuclear factor κB [33,34]. Published studies demonstrated that prophylactic enteral lactoferrin supplementation prevented late-onset sepsis and NEC in preterm neonates [32,33]. In contrast, the findings of a recent study revealed that lactoferrin enteral supplementation did not decrease incidence of NEC and infection [34].

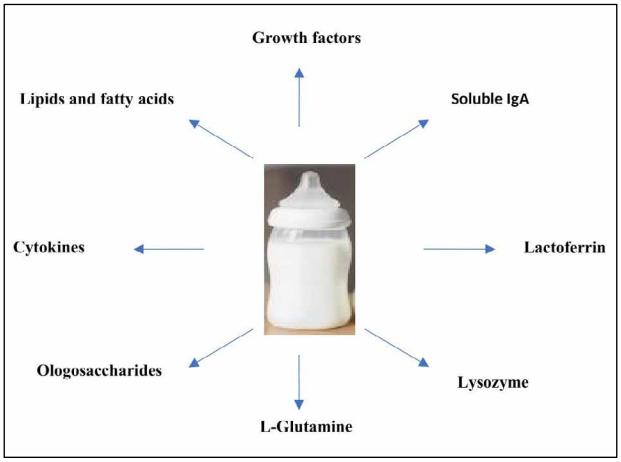

FIGURE 1. Summary of immunomodulating components of BM and protection against NEC.

TABLE 1. Immunomodulating components of BM against NEC.

Component	Role in NEC protection	Reference
Lactoferrin	a. acts against Gram-bacteria in the stomach b. synergistically with lysozyme kills Gram- bacteria in the stomach c. binds to the TLR4 and CD14 receptors blocking the adherence of bacteria to the intestinal epithelium d. promotes the apoptosis of infected intestinal epithelial cells e. stimulates the growth of commensal bacteria f. reduces the production of inflammatory cytokine IL-1β, TNF*-α, IL-6, IL-8	32-34
Lysozyme	a. synergistically with lactoferrin degrades the outer wall of pathogens bacteria protecting the intestine b. protects the intestinal epithelial. From NEC	35-36
Oligosaccharides	a. inhibit pathogens from adhering with epithelial cells of intestine b. preserve the growth of lactobacilli and bifidobacterial c. reduce the incidence of NEC	37-41
Cytokines *TNF-α, IL-6, IL-6, IL-12, IL-2, INF-γ, TGF-β, IL-7, IL-10, IL-18, G-CSF*	a. contribution in the pathogenesis of NEC b. anti-inflammatory properties (IL-10)	42-46
L-glutamine	a. stimulates intestinal cell proliferation and small bowel growth b. antioxidant, anti-apoptosis and anti-inflammation activities which are involved in the pathogenesis of NEC	47-50
Secretory IgA	a. entraps microbes in the mucus of intestine b. downregulates pro-inflammatory bacterial antigens on commensal bacteria	51-52
Lipids and fatty acids - Saturated and monounsaturated fatty acids - Long-chain Polyunsaturated fatty acids (LCPUFA)	a. promotes intestinal barrier b. regulate the intestinal inflammation	53-54
Growth factors *EGF, HB-EGF, IGF1/IGF2, VEGF, EPO, G-CSF*	a. maintain intestinal homeostasis b. protect intestinal barrier	55-57

TLR4: toll like receptor 4, CD14: cluster of differentiation 14, TNF: tumor necrosis factor, INF-γ: interferon gamma, TGF-β: tumor growth factor beta, G-CSF:granulocyte colony stimulator factor, EGF: epidermal growth factor, HB: heparin binding, IGF: insulin like growth factor, VEGF: vascular endothelial growth factor, EPO: erythropoietin

Lysozyme

BM lysozyme is an antibacterial immune-active enzyme, which in synergy with lactoferrin, binds to lipopolysaccharide in the outer wall of bacteria, resulting in degradation of internal proteoglycan matrices of bacterial membranes [35]. It has been also found that lysozyme is secreted by Paneth cells in the GIT in response to enteric pathogens [35]. Concerning NEC, it has been suggested that neonates with NEC have reduced concentration of Paneth cells [25]. As a result, the role of lysozyme in protecting breast fed neonates from intestinal inflammation and NEC is significant [36].

Oligosaccharides

The human milk oligosaccharides (HMOs) consistof three to 32 sugars in size, that they are not digestible by the human intestinal tract, and represent roughly 20% of the whole carbohydrate concentration of BM [37]. HMOs lie on the position of microbial receptors and inhibit pathogens from adhering with epithelial cell walls of the intestine. Also, it has been found that they preserve the growth of *lactobacilli* and *Bifidobacteria* in the gastrointestinal tract, and reduce the presence of pathogens [38]. Studies have shown that only *Bifidobacteria* and *Bacteroides* are able to consume HMOs by encoding the complex array of glycosidases necessary to transport and digest HMOs [39]. In a randomised control study of 75 preterm neonates (birth weight less than 1500 g and gestational age equal or less than 34 weeks), Armanian et al. [40] investigated the effect of enteral supplementation with a probiotic mixture of short- and long- chain oligosaccharides versus no intervention on incidence of NEC in preterm neonates fed exclusively BM. They noticed a reduced incidence of NEC in the group with probiotic supplementation. Moreover, it has been demonstrated that the concentration of HMOs

can predict NEC, as lower concentration is associated with higher incidence of NEC [41].

Cytokines

Cytokines represent protein hormones that interfere in both natural and specific immunity of the newborn infant. BM is the main source of cytokines, specifically anti-inflammatory cytokines, for neonates that are in general deficient of these proteins [42]. Cytokines have antimicrobial, anti-inflammatory and immunomodulatory activities, providing passive protection and modulating the immunological system of the host. Furthermore, cytokines include chemokines, which stimulate movements of other cells, interleukins and interferons [43]. The spectrum of cytokines of BM encompasses pro-inflammatory cytokines (Tumor Necrosis Factor-a, IL-6, IL-8, -1L-12, IL-2 and Tumor Necrosis Factor-γ) and anti-inflammatory cytokines (Transforming Growth Factor-β, IL-7, IL-10, Granulocyte -Colony Stimulating Factor) [42]. Maheshwari et al. [44] studied the level of cytokines in blood in extremely low birth weight neonates who develop NEC in early neonatal period, and found that the diagnosis of NEC was associated with elevated blood levels of IL-1β, IL-6, IL-8, IL-10, monocyte chemoattractant protein-1/CC, C-reactive protein, and lower blood levels of TGF-β, and IL-2. Emami et al. [45], in an experimental NEC study, investigated the anti-inflammatory properties of IL-10, and found an increase in its concentration, suggesting a protective role in the pathogenesis of NEC by weakening the degree of intestinal inflammation. Moreover, Wang et al. [46] investigated the levels of pro-inflammatory cytokines IL-1, and TNF-α and anti-inflammatory IL-10 in premature neonates with NEC and compared them with premature and full-term neonates without NEC. The results showed a statistically significant increase of the above-mentioned cytokines in patients who developed NEC, suggesting a role in the pathogenesis of NEC.

L-glutamine

L-glutamine is present in BM and can stimulate intestinal cell proliferation and small bowel growth, by supplying metabolic nourishment to intestinal epithelial cells. Studies in cells and experimental models indicated that L-glutamine exerts multiple biological activities such as antioxidants, anti-apoptosis and anti-inflammations, which are involved in the pathogenesis of NEC [47]. A lack of glutamine has been proposed to be a risk factor for NEC [48]. In addition, Pawlik et al. [49] reported a lower incidence of NEC after enteral administration of glutamine in a clinical study that included 106 very low birth weight premature neonates versus the control group. However, other studies showed no effect on the incidence of NEC in premature neonates after enteral supplementation with glutamine [50]. More studies are needed to investigate the beneficial intestinal effects of glutamine.

Breast milk secretory IgA

Secretory IgA (sIgA) are derived from the enteromammary and bronchomammary immune system and contribute to the defensive character of BM. BM sIgA has an immunomudulatory role in the GI as a result of entrapping dietary antigens and microbes in the mucus, or down-regulating the expression of pro-inflammatory bacterial antigenic determinants on commensal bacteria [51]. A recent study revealed the important role of BM IgA concentration, as IgA deficiency and reduced IgA-bound bacteria in the intestine was associated with increased risk of NEC development [52].

Breast milk lipids and fatty acids

The effect of BM lipids and fatty acids on gut development is not well-studied, but a lot of mechanisms have been suggested by which fatty acids may modulate the risk of intestinal injury and inflammation. Among different categories of fatty acids, long-chain polyunsaturated fatty acids (LCPUFA) have been reported to contribute to NEC prevention, because of their effect on intestinal barrier function and their critical role in regulating inflammation [53]. Several animal studies have shown that LCPUFA supplementation reduces NEC incidence, but despite these promising results, most current preterm human infant studies did not find any benefit of LCPUFA supplementation regarding the risk of NEC development [53,54].

Growth factors

BM growth factors, such as lactadherin, epidermal growth factor, heparin-binding epidermal growth factor and transforming growth factor-β2, have been reported to contribute to NEC prevention, by maintaining homeostasis of intestinal epithelium, facilitating intestinal mucosal barrier maturation or playing a role in GTI epithelium development pre- and postnatally [55,56]. More specifically, animal and human studies' findings about growth factors administration in NEC experimental models are promising and suggest a potential protective role, as decline incidence and severity of NEC [56,57].

Human-milk based fortifiers

Several studies indicate that BM alone provides insufficient nutrients for preterm infants and cannot meet the

increased demands for growth particularly in extremely premature newborns [58]. So, human milk fortification is currently recommended and widely accepted, in order to improve the nutritional profile of this population [58]. The recent availability of human-milk-based fortifiers overcame the concerns existing about the association between the administration of cow's milk-based fortifiers and NEC [59]. A recent meta-analysis of randomised control trials revealed that there was a reduction in the incidence of NEC with human milk-based fortifiers compared with cow's milk-based fortifiers [60]. However, further trials are required before this therapeutic strategy can be routinely implemented for preterm infants.

Human donor milk and prevention of NEC

Despite the fact that some immunomodulatory constituents of human milk are reduced after pasteurisation of donor milk, its beneficial effects are not completely lost. Although premature neonates receiving their own mother's milk have better feeding tolerance and lower incidence of NEC, there is no evidence that pasteurised donor milk does not administer some health benefits as does mother's own milk [61]. In a randomised trial, Schanler et al. [62] found an increased incidence of NEC in premature neonates fed with formula or donor BM compared with those premature neonates fed with their own mothers' milk. In addition, Christofalo et al [5] in a randomised controlled trial noticed that extremely preterm neonates who received bovine-based preterm formula showed significantly greater duration of parenteral nutrition and higher percentage of surgical NEC compared to those premature neonates fed with either donor human milk or human milk-based human milk fortifier. So, regarding the prevention of NEC, donor milk seems to maintain some of the immunological advantages of fresh human milk [63].

CONCLUSION

Prematurity and low birth weight due to prematurity remains a leading cause of neonatal mortality and morbidity. NEC is known to participate largely among gastrointestinal complications in this age group due to immature immunologic and host defense gastrointestinal systems. BM is considered as a tissue similar to plasma as it contains a variety of immunomodulatory components such as immunoglobulins, cytokines, lactoferrin, lysozyme and other factors that potentially deliberate protection against a diverse range of diseases. Despite ongoing research on the pathogenesis of NEC, the understanding of this devastating disease has increased slowly in recent years. Intestinal epithelial barrier, intestinal microbiota and the immature immunologic environment of the gastrointestinal tract have a crucial influence in the development of NEC, particularly in premature neonates. However, there is ongoing evidence that BM contains multiple components that aid to prevent, and modulate the immature immune system of premature neonates. From this point of view, BM is considered not only a choice of diet but rather a modulator of the immature immune system of the preterm neonate.

Conflict of interest: *None*

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