Pathogenesis of NEC: Impact of an Altered Intestinal Microbiome

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Introduction

Necrotizing enterocolitis (NEC), a disease most commonly seen in preterm infants, most often presents without warning and all too often rapidly evolves into a condition requiring resection of bowel and/or death. NEC has been largely recognized since the beginning of modern neonatal intensive care over 60 years ago[1,2]. There have been major efforts toward garnering a better understanding of this disease as well as its eradication, but it has persisted and is now one of the leading causes of mortality and morbidity in neonatal intensive care units[3-6]. There are several reasons for this lack of progress in understanding and eradication of this disease. These include a poor definition of what actually constitutes what is called "NEC". In fact, this entity probably represents several different diseases with different pathophysiologic pathways[2,7,8]. Even though a necrotic bowel is the final outcome of what we are terming NEC, the more proximal factors that lead to this intestinal necrosis may differ widely, and only the very distal components of the pathways appear to be common. Certainly those infants who have spontaneous intestinal perforations, those infants who have severe left-sided cardiac dysfunction leading to intestinal ischemia, and those infants who suffer intestinal necrosis with severe polycythemia and hyperviscosity should not be considered as having the same disease as a preterm infant who develops intestinal necrosis four weeks after birth and has clear signs of intestinal inflammation[9-14]. We need to also separate what we will subsequently term the "definition" of this disease versus the "diagnostic criteria" for this disease, since they are quite different.

The definition should include elements of the pathophysiology of the most classic form of this disease, which we will attempt to describe in this review. Diagnostic criteria should include specific, sensitive and accurate clinical and laboratory criteria that aid the clinician in recognizing the most classic form of this disease and differentiating it from entities such as spontaneous intestinal perforation, primary intestinal ischemia, sepsis and food protein intolerance, among several other entities. We will attempt to limit the this review to the more common, "classic" form of "NEC", which is seen primarily in preterm infants that is associated with inflammation and not associated with the congenital heart disease, hyperviscosity or spontaneous intestinal perforations. Since it appears likely that the introduction of certain types of foods to which the infant has sensitivity may also mimic the symptoms and signs of the more classic form of NEC, it may be difficult to differentiate this entity because of the large overlap in clinical presentation and likely sharing of several distal pathophysiologic pathways. Food protein induced enteropathy and cow's milk protein intolerance can present with bloody stools, and intestinal distress and some of these infants may actually exhibit pneumatosis intestinalis on the radiograph [15,16]. It is highly likely that this entity may also play a role in what is commonly diagnosed as NEC in preterm infants.

One of the most fascinating aspects of what we are learning about NEC is that appears to be related to host development. Most cases of NEC occur in preterm infants, but its onset does not relate to postnatal age as much as corrected postnatal age (based on last menstrual period or conception)[17-19]. Figure 1 illustrates what has been seen in several data sets with the post

conceptual timing of NEC (**Figure 1**) (Pammi et al unpublished data). From this it is obvious that NEC has a peak incidence at around 31 weeks post conceptual age. The reason for this peak and timing is poorly understood, but is reminiscent of the timing of retinopathy of prematurity, which also does not manifest until a similar post conceptual age, but depends on developmental stage of the retinal microvasculature. This suggests that host maturity factors are involved in the pathogenesis of NEC. Since not all preterm infants develop this disease, there are likely several other environmental factors that are involved and we will discuss some of the most likely of these factors, including intestinal dysbiosis.

The Concept of Dysbiosis

Although microbes were previously thought to play a role in the pathogenesis of NEC, the hypothesis for inappropriate colonization of the premature intestine as a major predisposing factor for the development of NEC was introduced by Claude and Walker[20]. This was based on the fact that "epidemics" of NEC had occurred, but no particular pathogen had been associated with NEC. The authors posited that rather than a direct infection, NEC may be the result of a secondary inflammation in response to microorganisms. This was based on the fact that histologic sections of intestine from infants with NEC often showed evidence of necrosis and inflammation in addition to bacterial overgrowth. They suggested a cycle of microbial induced neutrophil activation that can lead to inflammatory mediator release, vasoconstriction, and disruption of the intestinal barrier that was initially caused by in interaction of a "dysbiotic" intestinal microbiota and an immature intestine. They also suggested that various factors such as breast-feeding could reduce colonization by pathogenic organisms but induce colonization by commensal organisms, which in turn modulate inflammatory reactions and thus decrease intestinal injury.

Prior to the last decade, several studies have searched for a specific microbial origin using culture based techniques[21-23]. In addition to studies evaluating the potential bacterial origin of NEC using stool cultures, several studies suggested a viral etiology, based on findings that certain viruses were found residing in the resected or autopsy samples of intestinal tissues of babies who had developed the disease[21,24,25]. One of the difficulties faced in the studies related not only to the previously mentioned difficulty in defining and diagnosing NEC but also in the difficulty of the being able to culture various microbes that may have been associated with the disease. More recently, non-culture-based technologies have been developed, and with the advent of the human microbiome project these techniques have been applied to evaluating whether a certain microbiota is seen with NEC, which we will describe.

The Microbiome and NEC

Of the factors that predispose to the development of the most common form of NEC, factors that relate to an immature gastrointestinal tract pose the greatest risk: the majority of infants who develop NEC are preterm and it is also seen most often in babies who are born with the greatest

degree of prematurity[26,27]. Several studies have also suggested that there is a potential genetic component to the development of NEC. This is based the fact finding that there appears to be a slightly increased incidence of NEC in identical twins [28]. Human exome sequencing evaluating DNA from an infant who developed lethal NEC suggested the possibility of genetic variations related to this disease [29], but this variant and others [30,31] at this juncture have not yet proven to be major predisposing factors.

Perturbation of the intestinal microbiome and a hyper inflammatory response has been implicated in the development of NEC in preterm infants [32-34]. Compared to term infants, the intestinal microbiota of preterm infants has fewer bacterial species, less microbial diversity and increased proportion of potential pathogens [35,36]. Absence of microbial colonization of the intestine in germ free animals prevents the development of NEC in these animals [37,38]. Disturbance of the developing microbiome by antibiotics increases the risk of NEC in preterm infants [39,40].

b. Introduction to non-culture based studies (16S vs. metagenomics and other "Omics" technologies)

Culture-based techniques to evaluate the microbiome are restricted by their ability to culture most microbes that colonize the gut and falsely enriched by organisms that grow under laboratory conditions. Culture-independent molecular techniques have the advantage of probing organisms that cannot be cultured. Advances in molecular techniques, easy availability, decreased costs similar to those used in Human Microbiome Project [41,42] have spawned numerous studies. These techniques provide greater resolution of the microbial ecology for the evaluation of the neonatal intestinal microbiome.

Culture-independent techniques that are most commonly used are the gel finger printing methods [28,43] and sequencing the variable region targets of the microbial 16s rRNA gene (V1-V3 or V3-V5)[36,44,45]. Whole genome shotgun sequencing of microbial genes followed by assembly provides strain level resolution of the microbiome and also metabolic profiling of the microbial genes which provide a look at the metabolic function of these microbial communities. Combining "omics" technologies using rapidly developing bioinformatics is likely to provide systems based analyses of cascades that occur during the various stages of NEC pathophysiology. A recent metagenomic study in neonates with NEC suggested uropathogenic *E. coli* colonization as a risk for development of NEC [46]. Morrow *et al* investigated the association of the urinary metabolome and the intestinal microbiome and reported urinary metabolic predictors for the development of NEC[47]. Combining this with other "omic" technologies such as microbial and host cell transcriptomics is likely to provide a clearer picture of these pathogenic events leading to NEC.

c**. A primer on Microbial Host Interactions**

Another review in this volume buy Denning deals more extensively with the relationship between microbes and the innate immune system in the gastrointestinal tract. In brief, immune dysregulation in association with microbial dysbiosis has also been implicated in the pathogenesis of NEC. Excessive TLR4 signaling in response to LPS [37,48] and an exaggerated inflammatory response [33,34] in preterm infants have been implicated in the higher risk of NEC in preterm infants. In brief, various intestinal mucosal cells including the intestinal epithelium harbor receptors to microbial components. Chief among these are the toll like receptors (TLRs), which play a major role in innate immunity [37,49]. Activation of these Toll-like receptors result in signaling cascades that induce nuclear translocation of Nuclear Factor Kappa β (NFKβ), a transcription factor that induced transcription of various pro-inflammatory and anti-inflammatory cytokines and chemokines[34]. For example, activation of the chemokine IL-8 attracts neutrophils to trigger areas of the intestine, where they undergo phagocytosis, and other inflammatory responses which subsequently cause microvascular constriction, ischemia and injury to the intestine [50]. Certain microbes may also affect dendritic cells, which are of the monocyte/macro phage lineage. These cells can traverse the intestinal epithelium, grasp antigens from the lumen, carry them to the sub-epithelium and act as antigen presenting cells to undifferentiated lymphocytes in the intestinal lamina propria [51]. Depending on the surface receptors that are stimulated by the distinct microbial patterns these cells are exposed to, interaction between dendritic cells may result in the differentiation of T cells into effector versus tolerogenic cell types. The precise mechanisms of these interactions as they relate to NEC are currently poorly understood and are hampered by the fact that animal models used to study NEC largely depend on hypoxic ischemic injury and other stressors, large doses of proinflammatory mediators and/or chemically excising Paneth cells, and thus likely present very different pathways in terms of the proximal events that lead to the disease compared to those seen in the preterm infant. Interpretation of pathophysiology from these models in terms of accurate translation to the events incurred in human preterm infants need to be cautious.

Exposure to microbes and/or microbial components during prenatal life may play a role in the pathogenesis of NEC. Recent evidence suggests that the fetus does not reside in a sterile environment[52,53]. Rather, the fetal maternal unit is constantly exposed to microbes and microbial components and metabolites that may be arising from various sites of entry such as the vagina, gastrointestinal tract of the mother, and the mother's mouth. It is been known for over 30 years that the amniotic fluid, even without ruptured membranes is not a sterile medium. More recent studies have shown that amniotic fluid contains microbes that cultivatable on that can only be detected with non-culture based techniques[52,53]. Furthermore, the placenta harbors microbial DNA[54]. With the presence of microbes in the amniotic fluid, and the well-known fact that the fetus swallows large quantities, up to 150 mL per kilogram per day, of amniotic fluid and is highly likely that the fetal gastrointestinal tract is also exposed to large number of microbes, and microbial components during gestation. In fact, there are several studies that demonstrate that the meconium contains microbial DNA as well as live microbes[55,56]. This is highly suggestive of the

exposure of the fetal gastrointestinal tract to the aforementioned microbes coming from the amniotic fluid or perhaps even other hematogenous sources. Studies suggest that the composition of these microbes in amniotic fluid, meconium and placenta differ depending on gestational age of delivery. Some studies have suggested that the microbial milieu in the meconium of babies who subsequently develop sepsis and or NEC differs from those who do not[56].

Is a well-known phenomenon of microbial host interaction that certain microbial components such as lipopolysaccharide (LPS), lipoteichoic acid (LTA) or flagellin affect their commensurate TLRs if provided in low doses in such a manner that subsequently blunts ('pre-conditions" or provides "tolerance") a response to subsequent high level exposure to similar pro-inflammatory mediators [37,57-59]. Whether such phenomena are occurring in the preterm infant and whether this relates to the development of NEC is still poorly understood.

d. The intestinal microbial milieu prior to the diagnosis of NEC---what is known and what can we conjecture

An enumeration of microbes of stools from preterm infants using 16SrRNA sequencing, has determined that the microbiota progresses through a succession of the bacterial classes from Bacilli, to gamma-Proteobacteria, to Clostridia[60]. By the time the infants approach 33 to 36 weeks post conceptual age, anaerobes constitute a large component of the gut colonization. Early studies utilizing similar technologies suggested that relative increases in the phylum Proteobacteria when compared to other major microbial phyla such as Firmicutes, Bacteroides and Negativacutes are associated with the development of NEC[44]. This intriguing relationship suggests that these may become targets of microbial intervention against NEC but true causality has not yet been proven and will be a requisite in the determination of whether a certain individual microbe or microbial pattern is responsible for the disease. A version of Koch's postulates needs to be fulfilled for causality to be proven.

Here, we would like to take the opportunity to propose an updated, albeit simplistic version of how the microbial milieu of the intestine relates to the pathogenesis of NEC. We propose a genetic predisposition may be present in some cases, but stage of intestinal development in relation with the microbial milieu play a major contributing role that contribute to a "perfect storm" scenario for the initiation of NEC. Primary hypoxia-ischemia does not play a role in the pathogenesis of the most common form of NEC, as once thought[61]. Rather, the microvasculature under the control of vascular endothelial growth factor [62] is affected by stressors such as inflammatory mediators such as LPS found in cell walls of certain taxa of microbes (Proteobacteria) are sensed high levels of cell receptors (TLRs) which in turn signal NFKβ mediated cytokine/chemokine production with subsequent phagocytic neutrophil activation and tissue damage with an exaggerated inflammatory response (Figure 2).

e. Factors that alter the microbiome and may predispose to NEC (antibiotics, H2 blockers, human milk)

Several studies evaluating antibiotic use in preterm infants suggest an increased odds ratio in the development of the disease, which is directly related to the days of antibiotic usage [40,63,64]. Studies by La Rosa et al., and Murgas, et al. suggest a relationship between antibiotic use in preterm infants, the microbial composition of the gastrointestinal tract and the subsequent development of NEC[60,65]. Additional studies suggest that the acid-base environment of the gastrointestinal tract may also play a significant role in the development of NEC[66]. This also can relate to the types of microbes present in the gastrointestinal tract because they respond differently to the acid-base environment in terms of their growth. Studies in which a formula was acidified resulted in a lower incidence of NEC. Other studies show that the use of H2 blockers also increases the odds ratio for development of NEC[66,67]. Germane to this relationship is work that has shown that H2 blockers favor the proliferation of the Proteobacteria over Firmicutes in the gastrointestinal tract. This relationship is highly intriguing in light of the fact that the Proteobacteria to Firmicute ratios also are altered in those infants who subsequently develop NEC[68].

The well-known phenomena that babies receiving their own mother's milk have a decreased incidence of NEC is likely to relate to many factors in the milk including its macronutrient composition, the composition of polyunsaturated fatty acids, lactoferrin, various immune cells, immunoglobulins, and microbes[69-71]. The fact that human milk contains microbes that appear to be a personalized for each mother's own infant is a fascinating phenomenon that may also relate to the decreased risk of NEC in babies fed their own mothers milk. The fact that donor milk is usually pasteurized and thus devoid of cellular material as well as live microbes could be highly germane in this regard. Studies utilizing only donor milk in comparison to commercial formula have not overwhelmingly favored the donor human milk and the prevention of NEC. Some of the studies included in meta-analyses of donor milk versus formula are nearly 30 years old and it is difficult to discern the criteria utilized in these studies to diagnose NEC in these older studies.

Milk oligosaccharides increase the growth of Bifidobacteria and protect against infections[69,72-75]. Which of the numerous milk oligosaccharides ("prebiotics") are the most bioactive and which ones may play a role in the prevention of NEC remain areas of intense investigation.

Where do we go from here?

In the future, it will be critical to have improved technologies for both the prediction and diagnosis of NEC. Diagnostic biomarkers include ones that may not be specific for NEC such as the C-reactive protein, white blood cell count, and platelet count[76]. However, more specific biomarkers are being developed which include tight junction proteins such as claudin-3, intestinal epithelial cell proteins such as intestinal fatty acid binding protein (IFAB), both of which can be analyzed noninvasively in the urine[76,77]. More predictive biomarkers using the proteomic, metabolomic and genomic technologies are being evaluated. A novel "Electronic Nose" technology is highly intriguing[78], but studies that prospectively critically evaluate its predictive and diagnostic potential remain to be done.

The fact that certain microbial patterns as well and even single microbes are being found to be a highly associated with NEC is also intriguing. However the finding of an association is not adequate to directly base therapeutic interventions. Further, more rigorous studies of the causality will need to be done. These will need to be based on *in vitro* as well as *in vivo* techniques to recapitulate the disease in cell culture, and/or animal models. Once these are established, directed therapies using highly specific, antibiotics, bacteria phage therapies, probiotic or other microbial therapeutic techniques may be further evaluated for safety and efficacy[79,80].

Although several studies suggest a probiotic approach to NEC may be useful in its prevention, the most rigorously done studies to date are not supportive even though numerous meta-analyses suggest efficacy [81]. The fact that numerous different probiotics, which are very different in their character, have been utilized in these meta-analyses has been subject of the criticism. Furthermore, FDA based drug standards should be utilized if these agents are to be used for prevention of NEC. Whether fecal microbial transplant (FMT) like approaches such as those being used in the treatment of Clostridium difficile infections may be of benefit in the prevention of NEC could be a basis of future investigation[82]. Transfaunation of donor milk using small amounts of the baby's own mother's milk may also be a means to "personalize" the donor milk to provide a means to protect the infant's intestinal tract from the microbial environment that predisposes to NEC.

Summary

In this review we have discussed the microbial intestinal environment of the fetus as well as the preterm infant and have suggested that there this may play a role in the pathogenesis of NEC. However, this is an environmental factor that there needs to be taken into conjunction with the immaturity of the host gastrointestinal tract. It is very likely that the combination of these interacting factors play a major role in the pathogenesis of this a devastating the disease. The developmental pattern of innate immune responses in which certain TLRs (e.g., TLR4) peak during development along with blooms of certain TLR4 agonists shortly before the development of NEC is highly intriguing. The developing intestinal micro vasculature may also be similar to that in the eye. Thus this developmental stage of microvascular development may also play a significant intermediary role in terms of interaction between the intestinal microbial milieu, surface receptors and intestinal necrosis. Testing such hypotheses will be crucial in the future for our better understanding of NEC and the fact that the microbes present a likely target in the proximal cascade for the development of this disease holds promise for future intervention.

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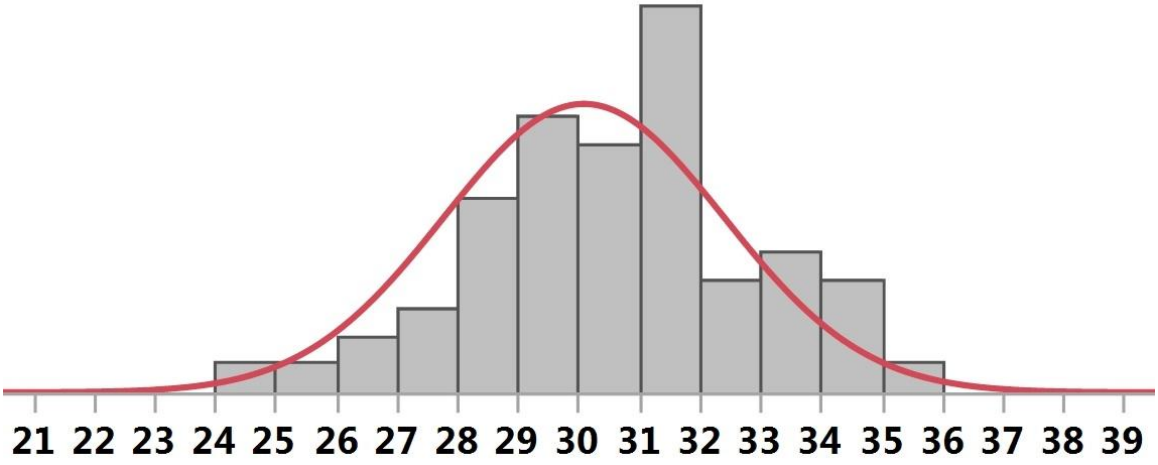
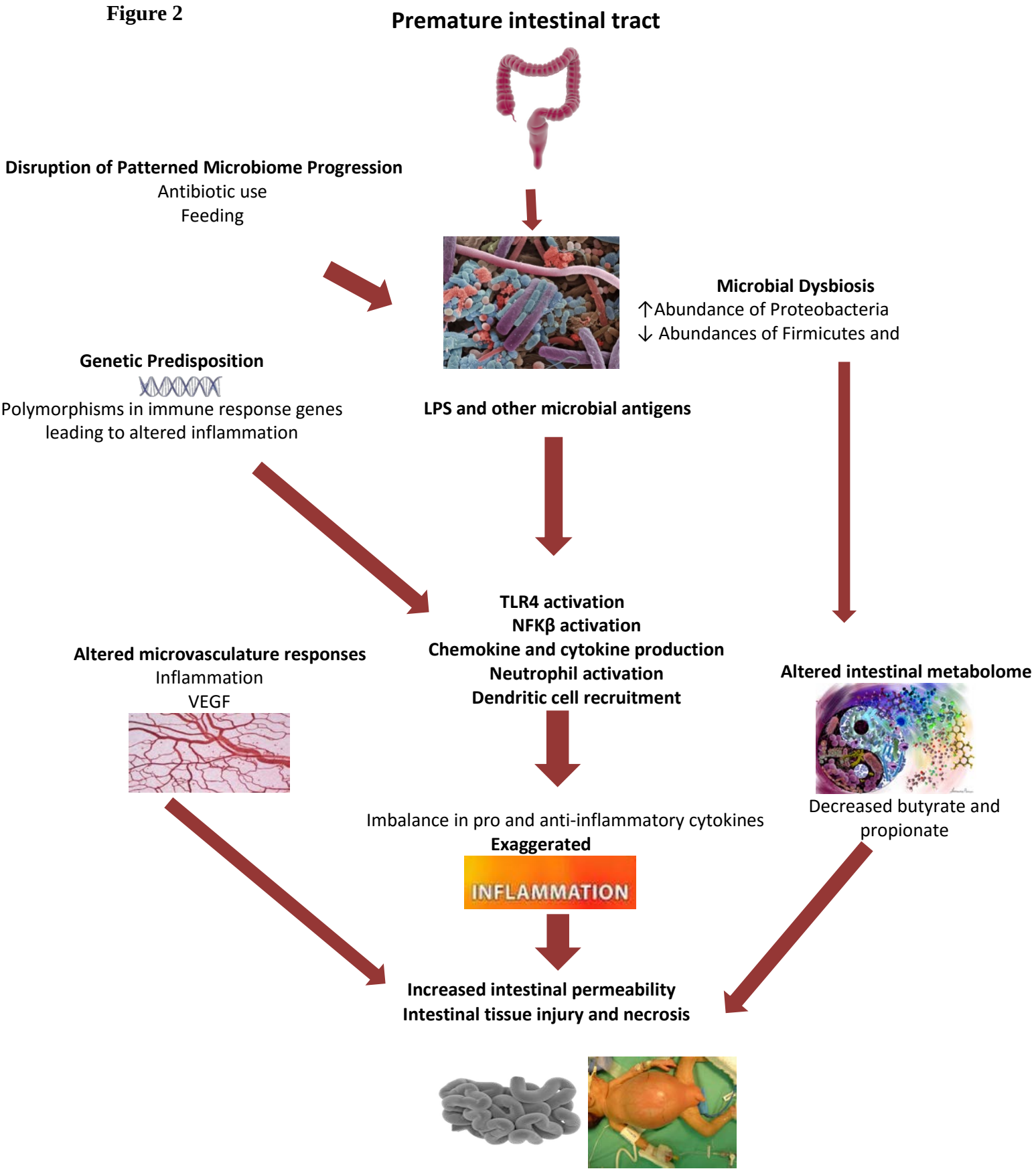


Figure 1: The X-axis, corrected gestational age is plotted against number of infants with NEC on the Y-axis and shows that the corrected gestational age of approximately 30 weeks represents the peak age of onset of NEC.

Figure 2


Premature intestinal tract
Disruption of Patterned Microbiome Progression
Antibiotic use
Feeding
Microbial Dysbiosis
↑Abundance of Proteobacteria
↓ Abundances of Firmicutes and
Genetic Predisposition
Polymorphisms in immune response genes leading to altered inflammation
LPS and other microbial antigens
TLR4 activation
NFKβ activation
Chemokine and cytokine production
Neutrophil activation
Dendritic cell recruitment
Altered microvasculature responses
Inflammation
VEGF
Altered intestinal metabolome
Decreased butyrate and propionate
Imbalance in pro and anti-inflammatory cytokines
Exaggerated
INFLAMMATION
Increased intestinal permeability
Intestinal tissue injury and necrosis
NECROTIZING ENTEROCOLITIS