Chapter 5

Microbes and the Inflammatory Response in Necrotising Enterocolitis

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Additional information is available at the end of the chapter

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1. Introduction

Necrotising enterocolitis (NEC) is a progressive disease of the neonatal intestine beginning in the distal ileum and proximal colon and characterised by inflammatory necrosis [1,2]. It typically affects low birth-weight, preterm infants who account for the majority (70–90%) of cases [3–5]. Since the 1960s, advances in medical care have raised the survival rate for preterm infants with increasingly shortened gestation periods, resulting in a concomitant surge in NEC cases. The overall incidence of NEC is generally accepted as ranging from <1% to 5% of neonatal intensive care unit (NICU) admissions, or up to 5 cases per 1,000 live births [4–6]. There is an inverse relationship between NEC and birth-weight, so that very low birth-weight infants (VLBW; <1500 g) carry the greatest burden of disease [4,5,7]. Caplan reported NEC rates for VLBW infants vary greatly across countries, ranging from 1.5% in Japan to 28% in Hong Kong, with racial disparity apparent in VLBW black infants who have an increased risk and greater associated mortality [5,8]. Despite advances in neonatal care, the overall mortality remains high at around 20–30% [3,8-10]. An estimated 20-40% of infants with NEC require surgery, which has a case fatality rate of up to 50%, the smallest, least mature infants having the worst prognosis [5]. Most cases of NEC are sporadic with no clear seasonal distribution, but outbreaks have been documented [5]. Treatment of NEC is mainly supportive with the administration of broad-spectrum antibiotics while surgery is indicated for intestinal perforation or removal of necrotic bowel segments. NEC complications and sequelae include serious neurodevelopmental delay, poor growth, intestinal obstruction due to scarring, short bowel syndrome, and liver failure due to prolonged hyperalimentation [6, 9]. The annual financial cost of NEC is considerable and in the USA has been estimated at $500 million to $1 billion [9].

1.1. Clinical classification

The classification system of Bell has historically proved important in defining three main stages: suspected, definite and advanced NEC [11]. Modifications to Bell's criteria have provided a more detailed system of clinical staging as shown in Table 1 [12,13]. However, Gordon et al and others have challenged the belief that NEC is a single entity, preferring to view it as an umbrella term for a number of separate diseases with some common features [13,14]. Although relatively uncommon, conditions, which mimic neonatal NEC, such as focal bowel perforation, intussusception, ecchymotic colitis, appendicitis and shigellosis, have been reported and may complicate the clinical diagnosis [14-18].

Stage (NEC)	Systemic signs	Radiographic findings	Intestinal signs
Stage I (suspected)	Temperature instability, apnoea, bradycardia	Normal or intestinal dilation; mild ileus	Gastric residuals, occult blood, mild abdominal distension
Stage II A (definite)	Temperature instability, apnoea, bradycardia	Intestinal dilation, ileus, focal pneumatosis	Blood in stools, prominent abdominal distension, absent bowel sounds
Stage II B (definite)	As above plus mild metabolic acidosis and thrombocytopenia	As II A plus portal vein gas, ascites	Abdominal wall oedema with palpable loops and tenderness
Stage III A (advanced)	As stage II B plus mixed acidosis, oligouria, hypotension, coagulopathy	As II B plus worsening ascites	Worsening wall oedema, erythema and induration
Stage III B (advanced)	As II A, shock, deterioration in vital signs	As II B plus pneumoperitoneum	Perforated bowel

Table 1. Modified Bell's staging for necrotising enterocolitis (adapted from Kliegman RM et al and Gordon et al [12,13])

1.2. Risk factors

So far, four major risk factors for NEC have been defined with prematurity being the most consistent. At 36 weeks gestation there is a sharp decrease in the incidence of NEC, supporting the concept that gut maturation provides significant protection against development of the disease [4]. Nevertheless, NEC in term and high birth-weight infants is not unknown, although the risk factors appear to be somewhat different [4,6,13]. The introduction of enteral feeds, particularly formula milk, and subsequent colonisation of the neonatal intestinal tract with bacteria are believed to be significant risk factors in the development of NEC [4,6,19]. Not only does formula milk lack the gastrointestinal protective, anti-inflammatory and maturation factors present in breast milk, it can be a source of *Cronobacter sakazakii*, a neonatal pathogen implicated in some cases of NEC [19]. Early research concentrated on the role of hypoxia and ischaemia, with the idea that the subsequent mucosal injury due to lack of oxygenation initiates NEC through promotion of bacterial translocation and the inflammatory cascade [1]. Many

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animal models of NEC have subsequently relied on reproducing this type of damage [5,20,21]. Kosloske et al suggested the 'dive reflex', whereby blood flow is diverted away from the GI tract to vital organs, as one mechanism of intestinal injury but current opinion, based on analyses of risk factors over several decades, favours a secondary role for hypoxia-ischaemia [5]. Nevertheless, the fact that NEC most commonly occurs in the distal ileum and proximal colon, the watershed areas of the mesenteric arteries, suggests inadequate or disordered blood circulation constitutes a risk factor in some circumstances [1,22]. Prenatal circulatory events, umbilical and aortic catheterisation with dispersion of small emboli and congenital heart disease have been linked to NEC, but occur in a minority of cases [2,4]. Preterm neonates are more susceptible to hypoxia and intestinal ischaemia than term neonates because of poor vascular resistance [1,4,22]. However, there is a stronger association between NEC, prematurity, enteral feeding and the presence of bacteria in the GI tract than hypoxia-ischaemia [1,5,22]. Overall, ischaemia/reperfusion injury seems to be more relevant to the development of NEC in term infants and those preterm infants with spontaneous intestinal perforations [23]. Yet hypoxia may exert a subtle effect by sensitising the intestinal epithelium to bacterial products and this is discussed in more detail in section 4.1 [24].

Recent evidence has linked blood transfusions with subsequent NEC in extremely premature infant [25–30]. The terms TANEC (Transfusion Associated NEC) and TRAGI (Transfusion-Related Acute Gut Injury) have been coined, referring to this association [25,31]. A recent meta-analysis examining evidence for the association concludes that recent transfusion is associated with NEC, and that transfusion-associated NEC has a higher risk of mortality than NEC which was not preceded by transfusion [25]. The reason for the association, and whether it is causal has not been elucidated. Prior to concern about TRAGI, Doppler studies have shown a decrease in neonatal superior mesenteric blood flow during and after blood transfusion [32]. The reason for this is not clear, but blood transfusion appears to have wide-ranging effects on haemodynamics, possibly as a result of changes in the microcirculation. Digestion of food requires a major increase in gut blood supply and it has been suggested that limiting or ceasing milk feeds before, during and after a blood transfusion in susceptible babies may decrease the risk of TRAGI. This has not been subjected to any systematic study and therefore remains speculative. The need for a co-ordinated approach to investigate the association between blood transfusion and NEC has been highlighted by Blau et al and there is an online world registry for TRAGI (www.tragiregistry.com) [31]. Any trial of an intervention for TRAGI prophylaxis would need large numbers, requiring a multicentre approach.

There are other characteristics of the preterm infant favouring the development of NEC. The combination of poor gut motility and under-production of mucous decreases clearance and increases exposure of the epithelium to potentially harmful components of the luminal contents [1,5,19]. Moreover, induction of foetal hypoxia can further reduce postnatal intestinal motility [1]. The lumen contents of preterm infants may be more acidic, due to inadequate digestion/absorption of nutrients and bacterial fermentation of undigested milk, or more toxic, as formula feeding elicits toxic bile acids [1]. Bacterial overgrowth, as indicated by a positive hydrogen breath test, is considered to be a further consequence of delayed transit time and may promote NEC through increasing bacterial translocation from the intestinal lumen into

the tissues or through exposure to high concentrations of bacterial antigens [33,34]. More recently, it has been proposed that genetic polymorphisms in the genes encoding the interleukin (IL) 4 binding receptor alpha chain and the chemokine IL-8 may also be a risk factor for NEC [35]. Intrauterine infection is another risk factor for NEC; the microorganisms implicated and proposed causality are discussed in section 2.3.

In the normal course of events, acquisition of the enteric microbiota begins during the birth process through the ingestion of bacteria of maternal origin. Breastfeeding, handling by the mother and exposure to environmental bacteria create further opportunities for gaining new species [6]. Colonisation takes place in the first few days of life and is influenced by a multitude of factors such as mode of delivery, type of feeding (breast milk or formula), gestation age, hospitalisation, the surrounding environs, maternal infection and antibiotic therapy, with mode of delivery and type of feeding considered the most significant [36]. Whereas breast fed infants are regarded as having an enteric microbiota rich in bifidobacteria, a more diverse microbiota, including potentially pathogenic groups such as *Clostridium* and *Enterobacteriaceae,* has been traditionally associated with formula fed infants. However, modern infant formulas more closely represent breast milk, shifting the gut microbiota towards beneficial species such as lactobacilli [19,36]. Breast-fed infants are less likely to develop NEC as breast milk contains many protective bioactives [1]. The initial neonatal microbiota consists of facultative anaerobic bacteria such as *Enterobacteriaceae*, enterococci, streptococci and staphylococci, which are present within days of birth [33]. These bacteria consume oxygen providing the reducing conditions required for the growth of obligate anaerobes, typically bifidobacteria, clostridia and *Bacteroides,* appearing one to two weeks later [33,36]. It is well established that the intestinal microbiota has a profound effect on gut health, influencing physiology and metabolism, as well as maturing the infant immune system and protecting against pathogens [19,33]. However, in the preterm infant, the normal succession of bacterial colonisers may be interrupted by the administration of broad-spectrum antibiotics, gut immaturity, acquisition of nosocomial bacteria in the NICU and placement of orogastric or nasogastric feeding tubes, resulting in a more restricted enteric microbiota with delayed colonisation with bifidobacteria and without the individual differences seen in the healthy, term neonate [6,33,37]. The relationship between delayed succession, bacterial overgrowth and NEC is discussed in more detail elsewhere in this chapter.

2. The role of microbes in NEC

The belief bacteria are crucial for the development of NEC stems from a number of clinical and experimental findings. In two studies totalling over 100 infants, Sántulli et al and Schullinger et al were the first to credibly establish bacterial colonisation of the neonatal intestine was a requirement for this disease [38,39]. NEC does not usually occur immediately post-partum but some days later, when feeding has usually commenced and there is ample opportunity for substantial intestinal colonisation. Early-onset NEC occurs in the first week and it is more often seen in term or near term infants with risk factors such as cardiac disease or severe placental insufficiency, whereas in infants of lower gestational age/birth-weight, NEC is delayed until

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13-32 days [40,41]. The reason for this difference is not entirely clear, but it may relate to a difference in pathophysiology, with bowel ischaemia being the predominant factor in early-onset NEC and cytokine priming being the predominant factor in late-onset NEC. The case for bacterial colonisation is further strengthened by the absence of NEC in ischaemic, ileal segments of germ-free rats and in infants who are stillborn [42,43].

Regardless of the initiating factors, pathological changes certainly involve bacteria as the intramural gas produced in pneumatosis intestinalis contains hydrogen of bacterial origin [44]. Demonstration of bacteria and bacterial DNA in the intestinal wall of resected segments from NEC infants supports this finding [45,46]. Epidemiological studies also indicate NEC has an infectious origin as it may occur in clusters of related cases which are amenable to infection control measures [47]. Moreover, prevention of NEC has been achieved through the administration of enteral antibiotics [48]. Bacteraemia and endotoxinaemia are frequent complications of NEC but are more likely to be sequelae rather than the actual cause [49,50]. Historically, there have been many proposals put forward regarding the aetiology of NEC (reviewed by Obladen [14]) but two main theories have emerged concerning the infectious component:

Specific pathogen theory

This theory relies on the existence of a hitherto undiscovered, single bacterial pathogen causing intestinal infection in susceptible infants.

Abnormal colonisation theory

Even in health, many members of the enteric microbiota can be considered to have pathogenic potential. When the balance between pathogenic and commensal species shifts in favour of the former, a chain of events is triggered in susceptible infants resulting in NEC.

2.1. Microbes implicated in the aetiology of NEC

Enteric anaerobes

Among the enteric anaerobes, *Clostridium* species are notorious for their proteolytic, saccharolytic, toxin and gas producing activities, making them ideal candidate microorganisms for the specific pathogen theory. Pederson et al emphasised the similarities between neonatal NEC and gas gangrene of the bowel, suggesting that ischaemic lesions were ideal sites for clostridial invasion, the anaerobic conditions of the bowel favouring conversion of the spores to toxin-producing bacteria [51]. Studying the histology of resected intestinal segments from NEC infants led these researchers to conclude *Clostridium perfringens* (*C. welchii*) type A was the most likely cause. Some early experiments on germ-free rats showed injection of *C. perfringens* spores into the intestinal wall induced pneumatosis intestinalis, whereas a variety of other intestinal bacteria did not [52]. Parallels were drawn with pigbel, an acute necrotising enterocolitis common in older children and adults in Papua New Guinea, caused by type C porcine *Clostridium perfringens* [52,53]. These and other findings focused attention on the clostridia for many years. Several investigations indicated *C. perfringens* was more commonly isolated from NEC infants prior to, and at the time of presentation compared to control infants [54–56]. However, negative findings include the long-term study of Dittmar et al where *C. perfrin-*

gens was present in only nine of 41 cases of NEC, with organisms mostly recovered from the peritoneal cavity rather than stool, while Gupta et al did not detect clostridia in any of 23 NEC cases [57,58]. Although it's unlikely *C. perfringens* is the specific pathogen in NEC, intestinal colonisation with this organism was observed to be associated with a more severe form of the disease, a finding supported by Kosloske and Ulrich, and Bjornvad et al in an animal model [45,57,59]. As the oxygen tension of healthy tissue is likely to inhibit growth of clostridia, pre-existing tissue necrosis may be exigent. The hypothesis that clostridial alpha-toxin plays a major role in NEC has never been proven [57].

C. difficile produces two large molecular weight toxins known to cause antibiotic-associated colitis. Somewhat surprisingly, colonisation with this microbe seems not to be a risk factor for NEC, probably because the neonatal bowel is tolerant to *C. difficile* toxins [55,60,61]. *C. butyricum* has been advocated as a cause of NEC but Blakey et al found it was not more common in patients compared to controls [55]. A novel *Clostridium*, designated *C.* 'neonatale' was purported to be the cause of an outbreak of NEC in a Canadian hospital but has not been reported since [62].

Other intestinal anaerobic genera have not been fully investigated, probably due to the difficulty of culturing under strict anaerobic conditions. Despite the prevailing view that non-sporing anaerobes are frequently absent in the intestinal tract of preterm infants, a DNA-based study indicated *Bacteroides* were abundant, and a review of anaerobic bacteraemia in a NICU carried out by Noel et al indicated anaerobic bacteraemia was frequently linked to NEC [63,64]. It is likely that further culture-independent studies will be able to better define the contribution these bacteria make to the pathogenesis of NEC.

Staphylococci

Coagulase negative staphylococci (CoNS) are commonly found in the stools of NEC infants and have been associated with significant disease [65,66]. Hoy et al noted their presence in duodenal aspirates of VLBW infants [10]. The role of staphylococcal delta(δ)-toxin was examined by Scheifele et al and Scheifele and Bjornson, who believed that toxin positive CoNS were enteropathic [67,68]. δ-toxin, a secreted protein with a detergent-like action, caused significant bowel necrosis in infant rats and was cytotoxic for fibroblasts in vitro. Moreover, it could be detected in the stools of infants colonised with δ-toxin producing CoNS [67,68].

We investigated 25 CoNS isolates from the stools of six NEC and six control infants in Dunedin Hospital NICU. A diagnosis of NEC was made on the basis of clinical indications and pneumatosis intestinalis or peritonitis on X-ray as described previously [69]. CoNS were identified using API ID-32 STAPH strips (bioMérieux). PCR primers for the δ-toxin gene were based on sequence data published by Tegmark et al and PCR conditions were as described by McIntosh [70,71]. Cell-free culture supernatants of the CoNS isolates and a δ-toxin producing *S. aureus* control were tested for cell damage (cytopathic effect; CPE) against the small and large intestinal tissue culture cell lines, Caco2 and HT29 [70]. The results, summarised in Table 2, suggest that the δ-toxin gene was frequently present in CoNS isolated from the infants (23 of 25 isolates) but that insufficient toxin was produced to cause a CPE in either of the cell lines tested compared to the *Staphylococcus aureus* control, which induced cell death. Reduced

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potency of exotoxins is a common feature of CoNS such as *S. epidermidis*, and probably explains their low virulence compared to *S. aureus* [72]. However, this may not always be the case. We found the culture supernatant of a single *S. epidermidis* isolate among 29 CoNS isolated from the skin and nares of University of Otago undergraduate students which could induce a cytopathic effect in the intestinal cell lines (Table 2). The toxic strain was positive for the δ-toxin gene by PCR and Southern hybridisation, although we lacked the specific antibody required to ultimately prove the cytopathic activity was δ-toxin mediated. The δ-toxin theory has been largely dismissed, but our research suggests there may be occasional strains of *S. epidermidis* producing significant amounts of toxin. The findings of Scheifele et al may reflect the dominance of such strains at a particular time in their NICU. There is one instance of a small outbreak of NEC and bacteraemia associated with a δ-toxin-producing methicillin resistant *S. aureus*, supporting the hypothesis that this toxin may contribute to the pathology of NEC providing it is produced in sufficient quantities in vivo [73].

Rejection of the δ-toxin theory does not preclude a role for CoNS *per se*, which are known to express a number of other virulence factors [74]. In addition, preterm infants exhibit deficiencies in immune responses to CoNS, suggesting they may cause more aggressive infections in this group compared to term neonates [75]. Large, relatively stable reservoirs of CoNS have been identified in the faces, ear region, axillae and nares of preterm infants, with smaller, less stable populations elsewhere on the skin, indicating the widespread presence of this group of bacteria and their easy access to the GI tract [76].

Subjects	**NEC infants (n=6)**		**non-NEC infants (n=6)**		**Students (n=19)**	
CoNS isolates tested	**n=15**		**n=10**		**n=29**	
Staphylococcus:	†δ +	‡CPE	δ +	CPE	δ +	CPE
S. epidermidis	11	0	6	0	15	1
Other	3	0	3	0	13	0

†δ + indicates presence of delta-toxin gene

‡CPE: cytopathic effect of CoNS culture supernatant on intestinal cell lines HT29 and Caco2.

Table 2. Presence of delta-toxin gene and cytopathic effect of coagulase-negative staphylococci colonies cultured from the stools of infants with and without necrotising enterocolitis and the skin/nares of university students.

Enterobacteriaceae

The *Enterobacteriaceae* family includes both classical enteric pathogens, such as *Salmonella*, *Shigella* and diarrhoeagenic *Escherichia coli*, and commensals inhabiting the large intestine. It is the commensals that are common in the enteric microbiota of neonates, including those who develop NEC. Despite their commensal status, these enterobacteria often possess specific virulence factors and are a common cause of extra-intestinal infection. There are early reports of a particular association between *Klebsiella, E. coli* and NEC, with isolation of the same organisms from the blood in some cases [77–79]. A number of subsequent studies have

demonstrated their predominance in the faecal microbiota and duodenal aspirates of NEC infants [3,10,58,80]. *Enterobacter cloacae*, *Klebsiella* spp. and *E. coli* appear to be the most common NEC-associated bacteria overall [3,69,77–79,81]. With the exception of *Cronobacter* (*Enterobacter*) *sakazakii*, which appears to be a special case, many other investigations have not shown a link between particular species or strains and NEC.

Characteristics of *E. cloacae* relevant to the pathogenesis of NEC include resistance to complement-mediated killing (serum resistance), adherence to and invasion of eukaryotic cells in vitro and chelation of iron [69,82]. In *Klebsiella* species, bacterial capsules are responsible for resistance to complement-mediated killing and phagocytosis by polymorphonuclear granulocytes as well as inhibition of macrophages. Differences in virulence between strains are believed to depend on the presence of repetitive sugar sequences in the capsule, which mediate lectin-dependent phagocytosis [83]. Adherence to eukaryotic cells and iron chelation are other virulence factors reported in *Klebsiella* [69,83]. More recently, a cytotoxin has been detected in *K. oxytoca* causing antibiotic-associated haemorrhagic colitis [84]. *E. coli* is notable for its ability to acquire virulence factors which endow it with diarrhoeagenic capacity, but such virulence factors are only occasionally present in isolates from the stools and duodenal aspirates of NEC infants [3,10,58]. *E. coli* cultured from the blood and stools of NEC cases have been shown to pass through epithelial cell monolayers in vitro and highly adherent strains induced NEC-like injury in a weanling rabbit ileal loop model. However, *E. coli* from NEC infants were not shown to be more virulent than those from matched control infants [85,86]. Commensal *Enterobacteriaceae* are not exclusively found in NEC infants, nor are they present in all cases, so they cannot be regarded as the specific pathogen for NEC [3,69,79]. Nevertheless, their frequent occurrence and possession of relevant virulence factors does allow for them to have an important role. Like many other investigators, we have been unable to show *Enterobacteriaceae* cultured from the stools of NEC infants harbour more virulence factors than those without, although the uncertainty regarding the pathogenesis of NEC presents a barrier to discerning essential pathogenic characteristics.

Cronobacter spp. are recognised opportunist pathogens of neonates causing bacteraemia and meningitis as well as being NEC associated. Epidemiological studies have shown powdered infant formula to be the source of the microorganism in many cases although other sources, such as the maternal birth canal are suspected [87,88]. A typical outbreak of *C. sakazakii* associated NEC is described by van Acker et al [89]. Neonates who developed NEC over a two-month period were fed milk formula contaminated with *C. sakazakii*, with a cessation of cases when the formula was removed. *C. sakazakii* is the most common *Cronobacter* species in neonatal infection but does not fulfil the criteria for the single, specific agent of NEC as it is infrequently isolated [87]. The high mortality rate in *C. sakazakii* infection has stimulated research into the virulence of this species, and this is discussed in a later section.

Classical enteric pathogens

Reports citing classical enteric pathogens such as salmonellae and shigellae as causes of NEC or NEC-like conditions are rare [18,90]. Enteric viruses such as norovirus, rotavirus, torovirus, and astrovirus are more frequently implicated. Some investigators consider norovirus to be an emerging pathogen in the NICU, with NEC representing a severe presentation of infection

[91,92]. Human astrovirus was reported by Bagci et al to be the cause of NEC in a subgroup of infants and torovirus was found to be more common in NEC compared to control infants by Lodha et al [93,94]. Rotavirus has been demonstrated in the stools of neonates from day 4 of life and its presence is considered a risk factor for NEC [95]. Echovirus type 22, renamed human parechovirus, is also considered to be an enteric pathogen, although causality has not been fully established [96]. Birenbaum et al detected this virus in an outbreak of diarrhoeal illness in a NICU with some patients exhibiting the clinical signs and symptoms of NEC [97]. Rousset et al noted the presence of coronavirus-like particles in gut tissue samples from NEC infants and proposed that secondary proliferation of anaerobic bacteria occurred in the gut wall following viral damage of the intestinal epithelium [98].

The advent of molecular techniques has facilitated the detection of viruses and it is likely that future investigations will better define the viruses associated with NEC. However, claims that viruses are *the* cause of NEC are not substantiated by the pathology, which strongly supports a role for bacteria. It is more likely that viruses predispose infants to NEC through damage to the epithelium, aiding translocation of the bacteria into the intestinal submucosa. In this regard, both rotavirus and norovirus infections have been shown to lead to epithelial barrier dysfunction through a reduction in sealing tight junctional proteins and an increase in epithelial cell apoptosis [99-101]. Which viruses are involved and how frequently they occur remains open to question as most studies failed to simultaneously investigate the bacteria present, which may have been the invasive organisms causing NEC. It is unlikely that viruses or classical enteric pathogens are the specific pathogens in NEC as they are not universally present. For example, in a comprehensive study of 27 NEC infants, Ullrich et al found common bacterial, viral and parasitic gastrointestinal pathogens were absent in all cases [102]. Gordon et al have proposed viral NEC is a separate disease with a lower mortality rate [13]. However, the difficulty of clinically distinguishing between viral and bacterial NEC, coupled with the likelihood that secondary bacterial invasion occurs, renders this argument more academic than practical.

2.2. Diversity and numbers

It is clear many of the bacteria forming part of the enteric microbiota have pathogenic potential and it has been suggested that when the balance between pathogenic and commensal species shifts in favour of the former, a chain of events is triggered in susceptible infants leading to NEC. A number of studies have sought to investigate this abnormal colonisation theory by identifying and quantifying enteric bacteria at the time of NEC presentation and comparing the results with a control group of healthy infants. However, there is evidence the intestinal ecosystem is altered by inflammation while the microbiota is restored after the inflammatory signal is dissipated [103]. This may be described as a 'chicken and egg' situation; it is unclear whether changes in the microbiota of NEC infants cause the disease or are a result of the inflammation. Of particular value are prospective investigations, as they have the potential to elucidate the microbiota associated with the initiation of NEC

The early, prospective study of Hoy et al was restricted to culturable bacteria, but nevertheless demonstrated considerable quantitative changes in the faecal microbiota preceding both

confirmed and suspected episodes of NEC, with a decline in some species up to 72 hours before clinical onset and the emergence of others, particularly *Enterobactericeae* [80]. Most human colonic bacteria are not amenable to culture, so molecular studies constitute a more promising line of enquiry. Techniques are mainly based on the gene encoding the 16S subunit of bacterial ribosomal RNA. This gene has hypervariable regions which are genus- and sometimes species-specific. Sequencing certain hypervariable regions of the 16S rRNA gene and comparing with sequences of known bacteria deposited in databases is now a common and accepted method of identification. Variations of this technique include the use of species-specific fluorescent 16S rDNA probes which bind to bacteria *in situ* (fluorescent *in situ* hybridisation; FISH), and 16S polymerase chain reaction denaturing gel gradient electrophoresis (16S PCR-DGGE) which separates 16S rDNA fragments from the different species present in a mixed community of bacteria.

A recent study encompassing both culture and molecular techniques has strengthened the argument that *Enterobacteriaceae* and staphylococci are important NEC-associated bacteria. In a prospective study, Stewart et al found differences in the gut microbiota of preterm infants who developed NEC or positive blood cultures. Certain Gram-positive genera, *Enterococcus* and *Streptococcus* were more frequent in health while *Enterobacter* and *Staphylococcus* were associated with disease. Moreover, these changes were evident before the onset of clinical symptoms [104]. Mai et al demonstrated the faecal microbiota of preterm infants was more heterogeneous one week before NEC diagnosis, with a low carriage of *Proteobacteria*. In contrast, blooms of *Proteobacteria,* a major phylum including the *Enterobacteriaceae* (*Gammaproteobacteria),* and a decrease in *Firmicutes* subsequently occurred preceding the onset of NEC by at least 72 hours [105]. Other community studies have contrasted the enteric microbiota in infants without NEC with that of NEC infants at the time of presentation. Smith et al analysed tissue samples from infants with fulminant NEC undergoing surgery. Using FISH, communities of bacteria in the excised tissue samples were investigated. *Proteobacteria* were present in most samples, in this instance dominated by *E. coli* and to a lesser extent by *Enterobacter*. Overall composition varied from infant to infant, with some infants showing a high diversity in bacterial species and others a low diversity. Clostridia, present in a few of the neonatal samples, were associated with pneumatosis intestinalis diagnosed histologically but this was considered to be a secondary effect. The study noted the presence of two more unusual bacteria, *Ralstonia* and *Propionibacterium,* in most samples. However, their ubiquitous nature and lack of dominance in the tissues suggests they were not the primary pathogens [106]. Wang et al profiled microbial communities in faecal samples from of 10 NEC infants and 10 non-NEC controls collected at the time of diagnosis. Limited microbial diversity was seen in all the preterm infants with a predominance of *Gammaproteobacteria.* Each infant had a unique microbiota (including twins) and no specific pathogen was implicated. However, differences between NEC and control infants were seen. Microbial community structure in the NEC infants was the least diverse but with a *Proteobacteria* bloom, which was absent in controls, even though the same bacterial species were present. NEC infants had received significantly more antibiotic treatments and it was hypothesised that antibiotics inhibit the bacteria which normally check the growth of *Proteobacteria* [107]. These findings are disputed by Mai et al, who found that

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overall microbial diversity at the time of diagnosis does not differ between healthy preterm infants and those diagnosed with NEC [105].

A confounding factor is that antibiotic therapy is frequently applied to preterm infants with the possible outcome that the reduced microbial diversity reported to be a feature of NEC could be a consequence of the antibiotic. Tanaka et al demonstrated antibiotic exposure in the pre- or early postnatal period greatly influences the enteric microbiota with arrested growth of beneficial bifidobacteria and overgrowth of *Enterococcus* and higher *Enterobacteriaceae* populations at one month of age [108]. Antibiotic use following preterm rupture of membranes (PROM) may alter the bacterial flora, and the effect of different antibiotic regimens in PROM has been examined with regard to the later occurrence of NEC. The Cochrane review by Kenyon et al found that only beta-lactam antibiotics (including Augmentin) showed an association with later NEC. The relative risk was 4.72 (1.57 – 14.23) compared to placebo, n = 1880. The duration of initial antibiotic treatment in the NICU has also been shown to affect the later occurrence of NEC in extremely low birth-weight (ELBW) infants [109]. Cotten et al used data from 5693 ELBW infants from multiple NICU services to examine the later NEC rate following initial empiric antibiotic use where the cultures proved negative. There were many other risk factors for NEC, some of which were also associated with the duration of antibiotic use, but multivariate analysis allowing for these factors demonstrated that duration of antibiotic use beyond 5 days was associated with an increased risk of NEC [110].

2.3. Significant microorganisms

Despite many attempts, the specific pathogen theory of NEC has not been proven. The strongest candidate organisms, commensal members of the *Enterobacteriaceae*, staphylococci and clostridia are not universally present in NEC infants and may be found in the enteric microbiota of infants without NEC. Clostridia are likely to be secondary invaders rather than primary pathogens. Molecular studies have not uncovered a previously unknown specific pathogen, although uncultured bacteria have been detected in stools of NEC infants [105,111]. The four criteria known as Koch's postulates, designed to establish causality between microbes and disease, are not fulfilled by the existing knowledge of NEC. Gupta et al, who undertook the first comprehensive case/control study of possible microbial causes, were the first to establish this fact [58]. Epidemics of classical enteric pathogens, including diarrhoeagenic *E. coli*, have been associated with NEC from time to time but most cases appear to be associated with normal members of the gut microbiota. *Mycoplasma hominis* and *Ureaplasma urealyticum* are associated with preterm birth but appear not to colonise the human gastrointestinal tract. Using PCR-DGGE, Millar et al were unable to detect these organisms in preterm infants with or without NEC [111]. The claim that enteric viruses cause NEC has not been substantiated although it seems likely that they represent a risk factor. The question of whether NEC infants are colonised by more virulent strains of the same species colonising infants without NEC has not been fully investigated but, as yet, there is little evidence to support such a hypothesis.

The abnormal colonisation theory, with its emphasis on community structure rather than specific organisms, has emerged as the most likely explanation for NEC. Possibly some members of the microbiota contribute to health while others increase the likelihood of NEC,

with quantitative changes heralding the onset. Even though they have never been proven to be the causative agents, *Enterobacteriaceae* are commonly found in the gut of infants with NEC and have been isolated from various sites in the body of affected infants [112]. The idea that a bloom of *Proteobacteria* precedes NEC is compatible with existing knowledge and highlights the importance of the Gram-negative *Enterobacteriaceae*. However, this probably does not occur in all cases. For example, Smith et al found Gram-positive bacteria dominated the faecal microbiota of NEC infants whereas a mixed microbiota of Gram-positive and Gram-negative bacteria occurred in the control infants [113]. Further investigation of CoNS is required, as these are a common component of the enteric microbiota in preterm infants and have been implicated in NEC. There is no convincing evidence that intestinal colonisation with enterococci is detrimental to the health of preterm infants. Rather, it may be beneficial, as some strains of *Enterococcus faecalis* have been shown to down-regulate the inflammatory response, modulate innate immune function in intestinal cell lines and to have a protective role in bacterial translocation [85,86,114,115]. In addition to the dominance of certain types of bacteria, NEC aetiology may involve the absence of other bacteria. For example, Blakey et al noted anaerobic *Bacteroides* spp. and lactobacilli were significantly less common in NEC infants compared to controls [55]. The paucity of probiotic bacteria in NEC infants and the phenomenon of microbial interference, where one species of bacteria inhibits another, are discussed elsewhere.

Several studies challenge the long held belief that the foetus is normally devoid of microorganisms and it now seems likely that colonisation begins before birth and can be detrimental. Intrauterine infection is a major cause of prematurity and is associated with adverse neonatal outcomes [116,117]. According to Gonçalves et al, microorganisms gain access to the amniotic cavity and foetus by four main pathways: (1) ascending from the vagina and cervix; (2) transplacental infection; (3) seeding from the peritoneal cavity via the fallopian tubes; (4) accidental introduction during invasive procedures [118]. The ascending pathway is probably the most common route of infection and a variety of microorganisms have been implicated [118]. PCR-based studies, such as that of DiGiulio et al, indicate microbial invasion of the amniotic cavity is common in the setting of preterm, pre-labour rupture of membranes and is underestimated using standard culture techniques [119]. *Mycoplasma* and *Ureaplasma* are the predominant genera in the amniotic cavity whereas NEC associated species are less frequently present [118,119]. Usually clinically silent in the mother, infection of the chorion and amnion are chronic, proinflammatory events believed to have wide-ranging, deleterious effects on the foetus [120]. Several investigations have reported an association between chorioamnionitis and NEC [116, 121–123], although others have failed to show any link [124,125]. A recent meta-analysis of 33 relevant studies revealed a significant association between clinical chorioamnionitis and NEC but there was no association where histological changes alone were the indicator of infection. A threefold increased risk of NEC was seen where chorioamnionitis diagnosed histologically was accompanied by foetal involvement [126]. Additional markers of inflammation, such as umbilical cord polymorphonuclear cell infiltration, *Ureaplasma urealyticum* colonisation and increased cord blood cytokine levels (IL-6, IL-8) have been linked to NEC. However, other studies have been unable to confirm these findings [126].

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The mechanisms underlying the relationship between intrauterine infection, the inflammatory pathway and NEC have not been elucidated but animal experimentation indicates intra-amniotic exposure to lipopolysaccharide or *Ureaplasma* induces intestinal inflammation in the foetus resulting in mucosal damage and impaired development of the intestine [127,128]. How closely these findings mirror the human situation remains to be seen. A contrary argument is that the foetal inflammatory response may be protective for neonates because it is already primed to deal with microorganisms interacting with the gut epithelium through the ingestion of microorganisms in amniotic fluid. Histologic chorioamnionitis is not necessarily associated with adverse long-term outcomes and may be protective for late onset sepsis [129,130]. On the other hand, if foetal inflammation is sufficiently damaging, it may predispose the neonate to NEC through impairment of the epithelial barrier. As noted earlier, despite the importance of intrauterine *Ureaplasma* and *Mycoplasma* as a risk factor for NEC, these microorganisms seem not to colonise the intestinal tract [111]. Other bacteria, mainly *E. faecalis*, *S. epidermidis* and *E. coli*, were the predominant species isolated from the meconium of healthy neonates by Jiménez et al who noted that bacteria can be detected in umbilical cord blood, amniotic fluid and foetal membranes in the absence of infection and inflammation [131]. Whether these antenatal gastrointestinal colonisers have a specific role in NEC has not been explored. That NEC occurs some days after birth suggests either additional microorganisms are required or the existing microorganisms need time to multiply in order to reach significant numbers.

2.4. Crossing the epithelial barrier

Structure and function

As previously mentioned, motility patterns in the small bowel are poorly developed in the preterm infant, particularly before 28 weeks gestation, with gastrointestinal transit times ranging from 8-96 hours compared to 4-12 hours in adults [132]. Gastric acid production and enterokinase levels are low in the premature infant, which may limit lipid and protein digestion in the small intestine, and together with lowered intestinal motility may be responsible for bacteria having substrate available for growth for longer periods. In 1990, Carrion and Egan investigated supplementing the feeds of premature infants with hydrochloric acid. The results were promising, but this approach has not been widely adopted, and appears not to have been investigated further [133]. It is postulated that bacterial fermentation of substrate (lactose) present in the infant gut damages the mucosa through the production of gas, which increases intraluminal pressure. The ability of some commensal species to ferment lactose is well known but there seems to be no correlation with NEC [58]. The surface of the gastrointestinal tract must allow entry of molecules that are beneficial to the host while at the same time preventing harmful microbes from crossing the barrier. Piena-Spoel et al observed increased intestinal permeability in human neonates with severe NEC, compared with control babies [134].

The intestinal epithelium from the stomach to the rectum is comprised of a single layer of polarised epithelial cells. The main functions of these cells are to absorb nutrients and also to prevent luminal bacteria and other antigens from crossing the intestinal barrier and entering the bloodstream [88]. Extrinsic barriers including gastric acidity, intestinal peristal-

sis and the mucus layer limit the access and adhesion of bacteria to the epithelial surface. The mucus layer is an organised extracellular matrix containing inorganic salts, non-specific antimicrobials and specific antimicrobial immunoglobulins, water and large glycoproteins (mucins) [135]. Mucins are produced by goblet cells within the crypts of the intestinal epithelium and released either constitutively or in response to infecting organisms [135]. Intrinsic barriers, which include the selectively permeable epithelial cell plasma membrane and the tight junctions that seal the intracellular spaces, block translocation of bacteria and restrict diffusion of macromolecules. Both these barriers are underdeveloped in the premature infant and this coupled with immaturity of the immune or cellular defense mechanisms may result in bacterial translocation leading to the inflammatory cascade resulting in NEC, even without prior injury of the mucosa. This hypothesis is supported by the fact that mice deficient in Muc2 have been shown both to be susceptible to infection and to develop intestinal inflammation. These mice, as well as having a deficiency in mucus production, had increased leakiness in the gut, which allowed microbes (both commensals and pathogens) to transit the mucosa [136]. Bergstrom et al suggest that the epithelium may be subsequently damaged either as a result of bacteria producing high concentrations of toxic metabolites or, alternatively, the presence of the bacteria stimulates recruitment of large numbers of polymorphonucleocytes to the site of infection resulting in epithelial cell death as neutrophils release cytotoxic mediators to control the infection [136]. The blooms of intestinal *Proteobacteria* seen prior to the onset of NEC in some infants may directly increase their rate of translocation *via* non-specific phagocytic uptake by epithelial cells lining the villi. Indigenous *Enterobacteriaceae* are considered to translocate with the greatest efficiency, *S. epidermidis* with moderate efficiency and obligate anaerobes with the least efficiency [137].

Tight Junctions

Tight junctions and adherens junctions are critical for maintenance of gut permeability and intestinal barrier function [138,139]. Tight junctions form a permeable barrier allowing the passage of fluids and solutes but not the other contents of the intestinal lumen. These junctions are made up of trans-membrane proteins (including occludins, claudins) and junctional adhesion proteins as well as cytoplasmic proteins (zona occludens - ZO-1, ZO-2, ZO-3) [140]. Using an epithelial cell monolayer (Caco2 cells) as in vitro model intestinal barrier, Han et al were able to demonstrate that proinflammatory cytokines interferon -γ, tumor necrosis factor - α and interleukin -1β could affect the expression of occludins and claudins involved in formation of tight junctions [141]. Another study demonstrated that epidermal growth factor prevented the disruption of tight junction proteins in an injury model using Caco-2 monolayers [142]. The importance of occludins and claudins in the formation of functional tight junctions has also been demonstrated in animal models of NEC where a positive correlation between ileal occludin mRNA levels and the progression of ileal injury was noted [143]. Erythropoietin (Epo), a component of human milk, has been suggested to have a physiological role in the developing gut. In vitro studies undertaken by Shiou et al, demonstrated that Epo is able to reverse the effect of IFN - γ and protect ZO-1 expression and barrier function [140]. In a rat model of NEC the same authors demonstrated that oral administration of Epo was able to reduce the incidence of NEC from 45% to 23%. If tight junctions are improperly formed or if

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they are damaged as a result of cytokine production in response to bacteria interacting with intestinal epithelial cells then bacteria will be able to translocate into the tissue causing some of the typical symptoms observed in NEC e.g. intramural gas. Reduced tight junction complexes have been associated with chronic inflammation in diseases such as ulcerative colitis and Crohn's disease. In these conditions there are often more bacteria found in association with the epithelium [144].

It is known that signals from the bacteria colonising the gut after birth play a role in maturation of physiological, anatomical and biochemical functions of the intestinal epithelial barrier [145]. Comparisons between conventional and gnotobiotic animals have demonstrated that the microbiota is involved in development, maintenance and repair of the intestinal mucosa [139,145]. As outlined previously, colonisation of the gut in neonates is influenced by gestation, postnatal age, environmental factors such as diet and the rearing environment, and administration of antibiotics. Cilieborg et al conclude that genetically determined gut characteristics (structure, function, immunity) and the time and mode of birth are the most crucial factors in the development of the enteric microbiota, which may be of a beneficial or harmful nature in terms of mucosal integrity [146]. *C. sakazakii* provides a good example of a potentially harmful member of the microbiota in that it is believed to trigger intestinal disease by modulating enterocyte-signalling pathways resulting in cell apoptosis [112]. This pathogen is also notable for its ability to induce the disruption of the tight junctions between enterocytes [147].

Mechanisms

The mechanisms by which classical enteric pathogens cross the intestinal epithelial barrier have been carefully studied as they provide putative targets for the prevention and treatment of infectious diarrhoeal diseases. Adherence is generally the beginning of the colonisation process leading to infection and, for invasive pathogens such as *Salmonella* and the NEC pathogen *C. sakazakii*, adherence is a prerequisite to internalisation in enterocytes. It is generally assumed that NEC-associated bacteria adhere to enterocytes prior to the development of the disease [148,149]. While direct evidence is lacking, animal studies support this contention and, from a theoretical point of view, it could be an essential step [85]. Adherence prevents bacteria being removed by luminal flow allowing them to deliver cytotoxic molecules, endotoxin and other pro-inflammatory substances directly to the epithelium. Features of the preterm intestine previously discussed such as scanty mucous, lack of secretory IgA and delayed colonisation with inhibitory probiotic bacteria certainly provide an opportunity for adherence of commensals to occur. When tested in an in vitro system, we found *Enterobacteriaceae* isolated from stool samples of NEC infants could adhere to both large (HT29) and small (CaCo-2) intestinal cell lines, although most did not adhere to the same extent as the diarrhoeagenic *E. coli* O111 control [69,150]. Such adherent bacteria may act as anchors for the formation of microcolonies on the mucosal surface, facilitating translocation through interactions with the innate immune system.

There is potential for bacterial cytotoxins to assist translocation of bacteria in the gut lumen through induction of enterocyte death. In a pilot study, we investigated 53 *Enterobacteriaceae* colonies cultured from the stools of four infants with NEC (at time of diagnosis) and four infants without NEC for cytotoxin production. The criteria for NEC diagnosis and selection of

NEC –ve infants were as specified by Brooks et al [69]. Cell-free, broth culture supernatants prepared from multiple bacterial colonies per stool sample were tested against a large bowel tissue culture cell line, HT29, as previously described [151]. Cytotoxic supernatants were diluted two-fold and re-tested to obtain the titre. All colonies were identified using API 20E biochemical test strips (bioMérieux). A small minority of the supernatants showed cytotoxic activity (Table 3), but there was no significant difference in their occurrence in NEC versus non-NEC infants or in the amount of toxin produced. Toxin producers were identified as *Serratia marcescens* (both groups), *Klebsiella oxytoca* (NEC positive group), *Escherichia vulneris* and *Klebsiella pneumoniae* ss. *pneumoniae* (NEC negative group). *Serratia marcescens* is known to produce pore-forming toxins, which may explain its cytotoxic activity for HT29 cells [152]. Cytotoxic activity in *Escherichia vulneris* and *Klebsiella pneumoniae* ss. *pneumoniae* is not recorded elsewhere. However, cytotoxin production in some strains of *Klebsiella oxytoca* has recently been described [153].

Infants	**NEC positive (n=4)**		**NEC negative (n=4)**	
Cytotoxin	Positive	Negative	Positive	Negative
Number of isolates	3	30	4	16
Toxin titre range	4–16		8–16	

Table 3. Cytotoxin production in *Enterobacteriaceae* from stool samples of infants with and without necrotising enterocolitis

3. Maturity of the gut and the innate immune response

As described in the previous section, the extrinsic barriers in the premature gut are not fully developed. Goblet cells, found throughout the intestine, are responsible for the secretion of mucus that protects the intestinal lining as well as providing some protections and nutrients for the bacteria that colonise it. There are both secretory mucins (Muc2) and membrane bound mucins (Muc3), which are co-secreted with trefoil factors (TFFs) [139]. The intestine can respond to injury by increasing mucin production. Resident microbes in the gut can also induce an increase in mucin production [154]. Mucins have been implicated in cellular signalling by virtue of the fact that they are able to develop binding sites for lectins, adhesion molecules, cytokines and chemokines. In the immature gut the coverage of mucin is scanty which may facilitate bacterial adherence to the epithelial cells. Paneth cells in the small intestine are able to secrete a wide spectrum of antimicrobial peptides against bacteria, fungi and viruses. Microfold (M) cells in the intestine sample the intestinal environment and deliver antigens to more specialised lymphoid tissue. Their role in disease in the premature neonate is unknown. However, impaired production of both MUC2 and TFF3 has been reported in clinical and experimental cases of NEC [139]. In a rat model of NEC Khailova et al demonstrated that when rats were given a probiotic bacterial strain *Bifidobacterium bifidum* that regulation of the mucin layer was observed with levels of mucin3 and TFF3 similar to those of control animals [139].

The innate immune system of the intestinal epithelium barrier has to be able to distinguish commensal bacteria from pathogens. Pattern recognition receptors on the intestinal epithelial barrier (transmembrane Toll-like receptors and intracellular nucleotide binding oligomerisation domain –like (NOD) receptors) have to be able to recognise microbial ligands (lipopolysaccharide, flagellin, lipotechoic acid, peptidoglycans and formylated peptides) known as microbial-associated molecular patterns (MAMPs). Depending on how the signal is perceived a number of responses can be generated - with commensal bacteria a protective response; with pathogenic bacteria an inflammatory response; or it can be a response that triggers apoptosis [145]. Commensal bacteria can dampen TLR-mediated inflammatory signals. The nuclear factor kappa B (NFκB) transcriptional control pathway has both anti-inflammatory and pro-inflammatory roles dependent on the microbial signal received [145]. Once the MAMP has bound to its respective TLR, the TLR triggers recruitment of the myeloid differentiation primary-response gene 88 (*Myd*88), which then recruits another protein known as IL-1R associated protein kinase. Activation of this gene leads to activation of NFκB and other regulators of gene expression. Along with the expression of inflammatory genes this is the basis of the innate immune response in the gut [155]. Receptors of the innate immune system are expressed on intestinal epithelial cells and also antigen presenting cells such as macrophages and dendritic cells. Discrimination of pathogenic bacteria from commensal bacteria is mediated by the trans membrane TLRs and the intracellular NOD isoforms [155]. The gut associated lymphoid tissue is made up of Peyers Patches, which can be described as mucosal lymph nodes overlaid with M cells. M cells have endocytic organelles that facilitate uptake of antigens from the intestinal lumen. Peyers Patches contain several types of antigen presenting cells such as dendritic cells and B cells. The lamina propria of the gut contains antigen presenting T cells, antibody secreting B cells, T cells and macrophages [156].

The microbiota, mucin and antibacterial products such as defensins and immunoglobulins help protect the host against pathogens. In infants with NEC who have a poorly developed gut with little mucin production and an abnormal microbiota compared to full term healthy infants, the potential for bacteria to cross the intestinal barrier and initiate inflammatory disease is much greater. Once we have a better understanding of the microbial-mucosal signalling components of inflammatory pathways and the regulation of these by commensal bacteria we may be able to find new ways of preventing NEC in premature infants. Alternatively, or as well as, damage to the intestinal mucosa by enteric viruses, harmful members of the gut microbiota, acidic or toxic luminal contents are likely to facilitate translocation. Injury due to hypoxic-ischaemic events in late-onset NEC is generally considered to be transient and unlikely to directly induce translocation. However, there may be subtle effects and when severe, it could be a permissive factor [20].

4. The inflammatory response

The initial step in any infection is the adherence of a microorganism to a host surface. As previously discussed, this binding may trigger a number of host responses such as chemokine and cytokine release, alterations in intracellular signalling pathways and induction of apop-

tosis. Cytokines play an important role in the regulation of inflammation. In cases of NEC several cytokines have been identified as being associated with the disease. A number of pro-inflammatory cytokines (IL-1β, IL-6, IL-12, IL-18 and TNF-α), an anti-inflammatory cytokine (IL-10) and platelet activating factor (PAF) have been associated with NEC pathogenesis and neonatal sepsis in both infants and in animal models of NEC [157–159].

Pro-inflammatory cytokines can cause increased production of nitric oxide which is known to modulate various physiological processes including inflammation [88,141,]. Nitric oxide is produced from arginine by nitric oxide synthases of which there are three isoforms one of which is inducible (iNOS). This inducible isoform is expressed at high levels during inflammation and is activated by cytokines such as gamma interferon and by bacterial lipopolysaccharide [88,160]. Nitric oxide may cause damage either directly or through its toxic intermediate, $ONOO^-$ resulting in a direct cytopathic effect on the cells (apoptosis) and inhibiting enterocyte proliferation and migration so that the intestinal mucosa cannot repair itself [88]. iNOS knockout mice have been shown to be more susceptible to infection with a number of microorganisms including *Porphyomonas gingivalis* and *Salmonella* [160]. Intestinal iNOS expression increased in the terminal ileum in a rat model of NEC with concurrent enterocyte apoptosis and decreased IL-12 production [161]. IL-12 is involved in bacterial clearance. Surgical specimens from infants with acute NEC have also demonstrated increased levels of iNOS and gamma interferon mRNA [157].

Platelet activating factor (PAF) has also been implicated in cases of NEC. This pro-inflammatory lipid mediator has been associated with intestinal mucosal injury and bowel necrosis in animal models of NEC and neonates with NEC [162,163]. A two-step enzymatic process is used to produce PAF [163]. Experimental animal model research has suggested that both PAF and intestinal bacteria are required to cause NEC as PAF alone cannot induce experimental NEC in a rodent model in the absence of the intestinal microflora [164,165]. PAF is released in response to hypoxia, infection or local injury and Soliman et al hypothesise that this then results in up-regulation of TLR4 in the intestinal epithelium allowing excessive bacterial activation of the intestinal inflammatory response [163]. Another group has also demonstrated that when PAF degrading enzyme is given in association with enteral feeding in a rat model of NEC that initiation of NEC is prevented [166].

IL-10 plays a protective role in the pathogenesis of NEC. Using wild type and IL-10 knockout mice Emami et al have demonstrated that in IL-10 deficient mice there is more evidence of epithelial apoptosis and dissociation of tight junctions compared to wild type animals [159]. In addition, when IL-10 knockout mouse pups were treated with IL-10 or phosphate buffered saline, pups given IL-10 had a greater rate of survival than the PBS treated pups and improved intestinal villus architecture. IL-10 can suppress the secretion of pro-inflammatory cytokines such as IL-2, TNF-α and gamma interferon. This cytokine is found in human breast milk and has been postulated to be one of the protective factors preventing the development of NEC. In addition, IL-10 can suppress expression of iNOS at the mucosal level in macrophages [167]. As described above, high levels of iNOS have been associated with cases of NEC. Lee and Chau have demonstrated that the enzyme heme-oxygenase -1 is induced by IL-10 and that this enzyme is required to mediate the action of IL-10 both in vitro and in vivo. When an HO

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inhibitor was given to mice, IL-10 mediated protection against LPS-induced septic shock was decreased [167]. IL-10 protection appears to be the result of down regulation of iNOS expression leading to less damage of the intestinal surface. However, if levels of HO-1 are reduced or absent IL-10 is not able to control iNOS expression. Further evidence for the role of NO in NEC has been provided by Ford et al who examined levels of inflammatory cytokines and NO in samples of intestine obtained from infants undergoing surgical resection for NEC and who demonstrated that NO was produced in large amounts by enterocytes in the intestinal wall leading to apoptosis of enterocytes in apical villi through peroxynitrite formation [157].

4.1. Signaling pathways

One of the newer approaches to understanding the pathology of NEC has been at a molecular level and examines the relationship between the intestinal epithelium and commensal bacteria. This research has identified a class of bacterial receptors known as Toll-like receptors (TLRs), in particular TLR 4, whose ability to respond to bacteria associated with the intestinal epithelium may in part explain why some premature infants are susceptible to NEC. More than ten TLRs have so far been identified in humans [168]. These receptors form part of the innate immune response and interact with different components of bacteria and viruses. TLR4, for example, is known to be the receptor for bacterial lipopolysaccharide. As NEC has often been shown to develop after gut colonisation with Gram-negative strains of bacteria, a putative role for TLR4 in the pathogenesis of this disease has been suggested. Mice with mutations in TLR4 or lacking TLR4 do not develop NEC [24,165]. Activation of enterocyte TLR4 leads to increased death of cells in the intestine through the mechanism of apoptosis [169]. TLR4 activation results in stimulation of IL-1R kinase via adaptor molecules MyD88 and MD2 resulting in activation through NFκB and up-regulation of pro-inflammatory cytokines [24]. Gribar et al have demonstrated that TLR4 expression is higher during gestation in the mouse and falls off shortly before birth [170]. They have also postulated a link between TLR4 levels and TLR9 levels in the pathogenesis of NEC. TLR9 recognises bacterial DNA as opposed to LPS recognised by TLR4. TLR4 levels are increased in the bowel of infants with NEC compared to control bowel samples [24]. In a mouse model of NEC, Leaphart et al demonstrated that physiological stressors such as hypoxia and LPS associated with the development of NEC sensitise the epithelium to LPS through the up-regulation of TLR4 and that if animals had a mutation in TLR4 the severity of NEC was reduced due to increased healing capacity of the epithelium [24]. Thus the effect of TLR4 appears to be two-fold by promoting damage to the small intestine through up-regulation of inflammatory cytokines and in reducing mucosal repair. As reported in the last section, there is also a known relationship between TLR4 up-regulation and PAF, one of the molecules implicated in the pathogenesis of NEC.

The relationship between TLR4 and TLR 9 and the signalling from both these receptors has an important role to play in the development of NEC [170]. In cases of NEC, in both humans and mice, increased TLR4 and decreased TLR9 expression was measured. TLR9 recognises CpG motifs of bacterial DNA. Bacterial DNA differs from human DNA in that is enriched with CpG motifs and is largely unmethylated [170]. Using a murine model of NEC, Gibrar et al demonstrated that NEC occurs when there is increased TLR4 and decreased TLR9 expression in

developing intestinal mucosa. Furthermore, they were able to show that activation of TLR9 with CpG-DNA inhibited TLR4 mediated signalling in enterocytes. The mechanism of inhibition was dependent upon the inhibitory signalling molecule IL-1R kinase. When CpG-DNA was administered to newborn mice the incidence of experimental NEC was significantly reduced. Other studies have also implicated TLR2 in the pathogenesis of NEC. TLR2 mRNA expression was increased along with TLR4 mRNA and activated NFκB in a neonatal rat model of NEC 48 hours prior to lesions being identified histologically [171,172]. TLR2 mediates host response to Gram-positive bacteria and yeasts through the NFκB pathway [173].

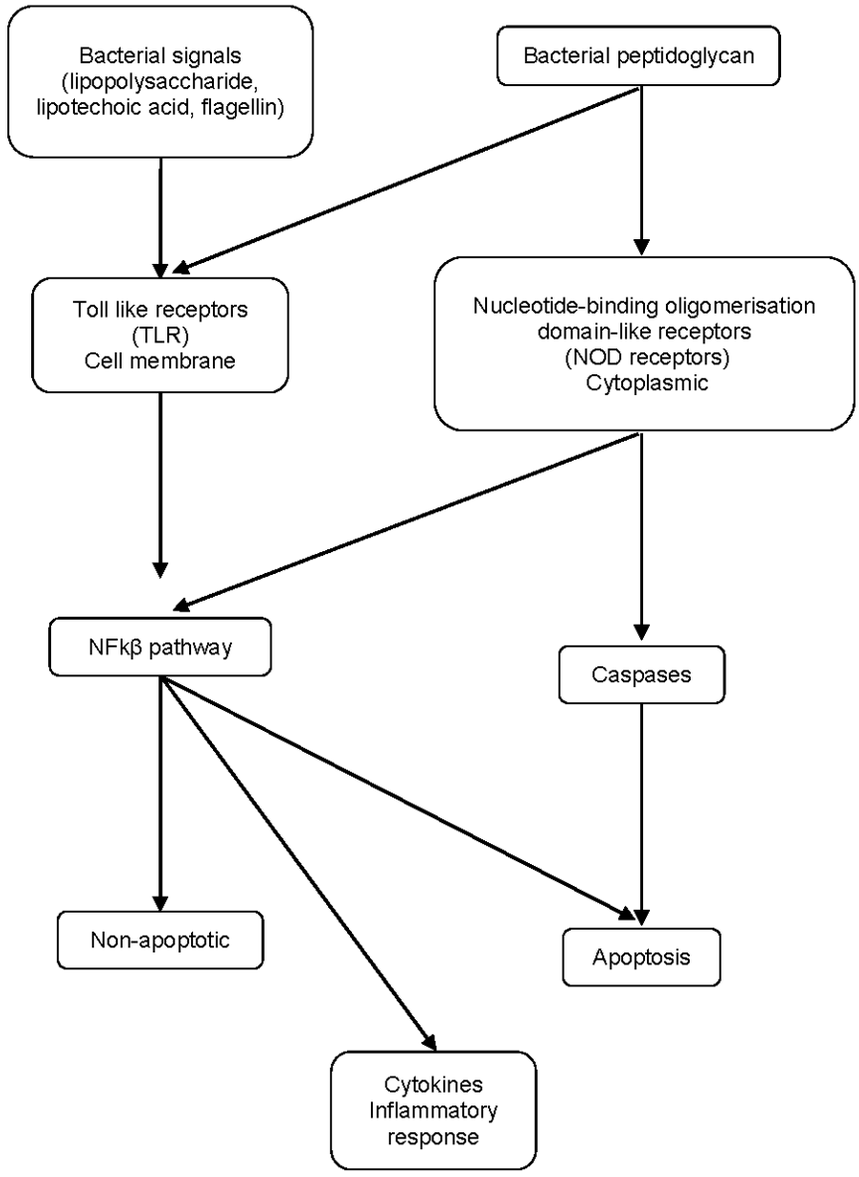

Figure 1. Effects of microbial stimulation on cellular outputs. Toll-like receptors (TLR) and nucleotide-binding oligomerisation domain-like (NOD) receptors recognise patterns of bacterial signals. The outcome of this interaction is variable depending on the commensal or pathogenic status of the bacteria.

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Signalling pathways of the innate immune system therefore play an important role in the development of NEC. Understanding of the interactions in this system may lead to the development of new therapeutic treatments for NEC e.g. probiotics with known effects on signalling pathways, anti-inflammatory molecules.

4.2. Cell damage

When pieces of bowel are removed during surgery for NEC and examined histologically, a large number of apoptotic nuclei signifying programmed cell death are observed in the tissues [157]. Studies using an animal model of NEC have demonstrated that apoptosis occurs prior to gross histological damage. When apoptosis was prevented using caspase inhibitors, the development of NEC was significantly reduced [174]. Also, the neonatal pathogen *C. sakazakii* is known to induce apoptosis in enterocytes. As the predominant anatomic lesion of NEC is coagulative or ischaemic necrosis, the contribution of apoptosis requires some explanation [2]. Necrosis is a term currently used by cell biologists for non-apoptotic, accidental cell death and by pathologists to denote the presence of dead tissue or cells regardless of how they have died. In the absence of efficient phagocytosis, apoptotic bodies may lose their integrity and proceed to secondary or apoptotic necrosis. Thus the presence of necrosis indicates cell death has occurred, but does not necessarily indicate how [175].

5. Hypothetical model of NEC

NEC is a complex disease influenced by several risk factors that may act alone and together. The innate immune system appears to play a large role in the establishment of NEC. We propose that in the preterm infant susceptible to NEC, scanty mucus production allows interaction between commensal bacteria and TLR triggering the innate immune response leading to loosening of intercellular tight junctions, enterocyte apoptosis and necrosis, resulting in translocation of bacteria (Figure 2a). Additional factors may also increase translocation, including infection with enteric viruses, presence of microbial cytotoxins or other toxic substances, and a bloom of certain bacteria in the gut lumen causing increased phagocytic uptake by enterocytes. In comparison, the intestinal epithelium of the healthy term neonate secretes adequate mucus, trapping enteric bacteria in the upper layers. In addition, the presence of sIgA and other antimicrobial substance inhibit bacterial colonisation of enterocytes, and any commensal bacteria that do adhere are recognised as not harmful, dampening TLR-mediated inflammatory signals (Figure 2b). In essence, a pivotal event in the development of NEC may be whether the innate immune system of the preterm infant intestine views bacteria reaching the enterocyte surface as friend or foe.

6. Probiotics

Microbial succession ensures that the intestines of healthy neonates are readily colonised with probiotic bacteria such as *Bifidobacterium* and *Lactobacillus.* In contrast, colonisation with these

bacteria is delayed in preterm infants [6]. Probiotic bacteria are avirulent, generally of human origin, and purported to have a number of beneficial effects. They are used both prophylactically and as a treatment for certain infections but claims they can colonise the bowel are more controversial, probably because this depends on the specific probiotic. Enteric probiotics may reduce the incidence and severity of diarrhoea due to enteric pathogens through competitive inhibition and/or production of antimicrobial substances. They have been shown to attenuate nitric oxide production, increase antioxidant activities, improve the mucosal barrier, upregulate anti-inflammatory and downregulate pro-inflammatory responses [6-8,176]. Additional probiotic effects, such as regulation of apoptosis and prevention of intestinal injury by *C. sakazakii* have been demonstrated in animal models of NEC [6]. All of these effects are relevant to the prevention of NEC and suggest that probiotics are the ideal, low-cost intervention for preterm and low birth-weight infants. The main difficulty in assessing the efficacy of probiotics for the prevention of NEC is that different bacterial species or strains with different probiotic effects are used in each study, so that the optimum probiotic for NEC, and the biological characteristics that it should possess, have not been well defined.

Trials of probiotics in preterm infants have had varying results. Those reporting a reduction in NEC have often employed *Bifidobacterium*, a dominant genus in the intestines of healthy infants, with or without other probiotic genera [177]. Efficacy in a rat model of NEC has also been demonstrated [139]. Administration of *Bifidobacterium* together with *Lactobacillus* is known to promote the growth of indigenous lactic acid bacteria through the production of short-chain fatty acids [178]. Administration of *Lactobacillus* alone does not appear to prevent NEC and Deshpande et al caution against its use [179]. In the NICU at Dunedin Hospital, Infloran, a probiotic mix of *Bifidobacterium infantis* and *Lactobacillus acidophilus*, is prescribed for infants <1500 g. It has performed well in trials, reducing the incidence of NEC by more than 50% [8]. A recent meta-analysis demonstrated that prophylactic probiotic therapy reduced the incidence of NEC by 30% overall, confirming the significant benefits of supplementation [179]. The risk that in critically ill neonates, or those with compromised gut integrity, probiotics may translocate into the bloodstream has been considered and this is an area requiring further investigation [178,180]. Nevertheless, probiotics are regarded as a promising approach for the prevention of neonatal NEC [7,8].

Cario et al demonstrated that TLR -2 was able to control mucosal inflammation in both in vivo and in vitro models by preserving TJ associated barrier assembly against stress-induced damage via MyD88. When colitis was induced in wild type mice using dextran sodium sulphate followed by treatment with a TLR-2 agonist, clinical signs of colitis were abrogated in all animals when compared to mice which didn't undergo treatment [181]. The probiotic *Bifidobactrium bifidum* was shown to up-regulate expression of TLR-2 in the ileum in a rat model of NEC and this was associated with significantly reduced epithelial apoptosis [139].

Whether probiotic therapy in preterm infants should include supplementation with prebiotics is open to question. Prebiotics promoting the growth of bifidobacteria and lactobacilli are naturally present as oligosaccharides in human milk [8]. The few existing trials of prebiotics in preterm infant have indicated an increase in stool counts of bifidobacteria and lactobacilli

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occurs. However, until more data is available, routine feed supplementation with prebiotics or synbiotics (probiotic, prebiotic mixtures) is not recommended [6].

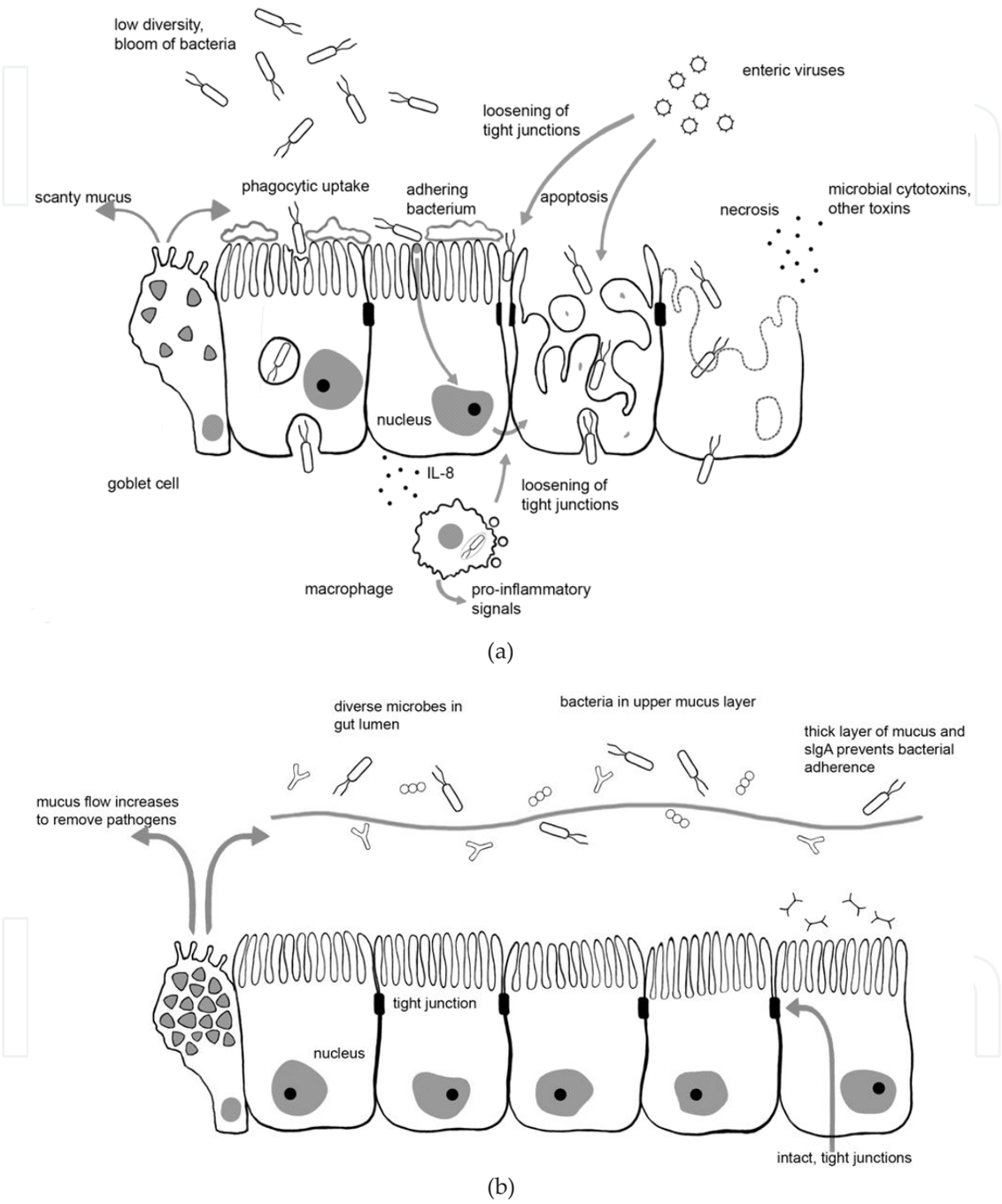

Figure 2. a) Progression of events leading to necrotising enterocolitis in the preterm infant. A bloom of bacteria due to low intestinal motility increases phagocytic uptake. Scanty mucus and reduced antimicrobial factors allow bacteria to adhere to enterocytes, activating NFKB via TRL leading to enterocyte apoptosis, necrosis and loosening of tight junctions. Bacterial translocation may be increased by presence of enteric viruses or microbial and other toxins. b) Intestinal epithelium of the healthy, term neonate. Diversity of enteric microbiota and normal gut motility prevent a bloom of one type of bacteria. Thick mucus, sIgA and other antimicrobial factors inhibit bacterial adherence.

7. Future directions

An article rather pessimistically entitled 'Necrotizing enterocolitis – 150 years of fruitless search for the cause' was published in 2011 [14]. We believe the search has been fruitful and a fuller picture of NEC is now beginning to emerge. NEC is a far more complex disease than early researchers anticipated, involving intrinsic gastrointestinal barriers, the innate immune response, signalling pathways and bacterial colonisation patterns. It seems likely that in individual cases of NEC there will be differences in both the aetiology and pathogenesis. Research into this disease will always be hampered by the fragility and vulnerability of the patients and ethical considerations, leading to a greater dependence on in vitro and animal model experimentation than is usual. Although necessary, antibiotic therapy is often a confounding factor in NEC research because it may eliminate the microbes that initiated the infection. Prospective studies represent a promising avenue of research especially when combined with DNA or RNA based methods for studying changes in the enteric microbiota, initiation of the inflammatory pathway and cell signalling. Through increasing our understanding of NEC, new targets for intervention will be identified. Further investigation of the mechanisms of action of probiotic bacteria will hopefully identify the ideal probiotic for NEC prevention. There is every reason not to be pessimistic about our ability to treat, and more importantly to prevent, this potentially devastating disease of preterm infants in the future.

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References

[1] Schnabl KL, Van Aerde JE, Thomson AB, Clandinin MT. Necrotizing enterocolitis: a multifactorial disease with no cure. World J Gastroenterol 2008;14:2142–2161.

[2] Hsueh W, De Plaen IG, Caplan MS, Qu X-W, Tan X-D, Gonzalez-Crussi F. Neonatal necrotizing enterocolitis: clinical aspects, experimental models and pathogenesis. World J Pediatr 2007;3:17–29.

[3] Boccia D, Stolfi I, Lana S, Moro ML. Nosocomial necrotizing enterocolitis outbreaks: epidemiology and control measures. Eur J Pediatr 2001;160:385–391.

[4] Noerr B. Part 1. Current controversies in the understanding of necrotizing enterocolitis. Adv Neonatal Care 2003;3:107–120.

[5] Lin PW, Stoll BJ. Necrotising enterocolitis. Lancet 2006;368:1271–1283.

[6] Stenger MR, Reber KM, Giannone PJ, Nankervis CA. Probiotics and prebiotics for the prevention of necrotizing enterocolitis. Curr Infect Dis Rep 2011;13:13–20.

[7] Wu S-F, Caplan M, Lin H-C. Necrotizing enterocolitis: old problems with new hope. Pediatr Neonatol 2012;53:158–163.

[8] Caplan MS. Probiotic and prebiotic supplementation for the prevention of neonatal necrotizing enterocolitis. J Perinatol 2009;29:S2–S6.

[9] Neu J, Walker WA. Necrotizing enterocolitis. N Engl J Med 2011;364:255–264.

[10] Hoy CM, Wood CM, Hawkey PM, Puntis JWL. Duodenal microflora in very-low-birth-weight neonates and relation to necrotizing enterocolitis. J Clin Microbiol 2000;68:4539–4547.

[11] Bell MJ, TernbergJL, Feigin RD, Keating J, Marshall R, et al. Neonatal necrotizing enterocolitis: therapeutic decisions based on clinical staging. Ann Surg 1978;187:1–7.

[12] Kliegman RM, Walsh MC. Neonatal necrotizing enterocolitis: pathogenesis, classification, and spectrum of illness. Curr Probl Pediatr 1987;17:213–288.

[13] Gordon PV, Swanson JR, Attridge JT, Clark R. Emerging trends in acquired neonatal intestinal disease: is it time to abandon Bell's criteria? J Perinatol 2007;27:661–671.

[14] Obladen M. Necrotizing enterocolitis – 150 years of fruitless search for the cause. Neonatology 2009;96:203–210.

[15] Khan A, de Waal K. Pneumoperitoneum in a micropremie: Not always NEC. Case Rep Pediatr 2012;doi:10.1155/2012/295657.

[16] Canioni D, Pauliat S, Gaillard JL, Mougenot JF, Bompard Y, Berche P, et al. Histopathology and microbiology of isolated rectal bleeding in neonates: the so-called 'ecchymotic colitis'. Histopathology 1997;30:472–477.

[17] Carman J, Grünebaum M, Gorenstein A, Katz S, Davidson S. Intussusception in a premature infant simulating necrotising enterocolitis. Z Kinderchir 1987;42:49–51.

[18] Sawardekar KP. Shigellosis caused by *Shigella boydii* in a preterm neonate, masquerading as necrotizing enterocolitis. Pediatr Infect Dis J 2005;24:184–185

[19] Emami CN, Petrosyan M, Giuliani S, Williams M, Hunter C, Prasadarao NV, et al. Role of the host defense system and intestinal microbial flora in the pathogenesis of necrotizing enterocolitis. Surg Infect (Larchmt) 2009;10:407–417.

[20] Luo CC, Shih HH, Chiu CH, Lin JN. Translocation of coagulase-negative bacterial staphylococci in rats following intestinal ischemia-reperfusion injury. Biol Neonate 2004;85:151–154.

[21] Zhou W, Zheng XH, Rong X, Huang LG. Establishment and evaluation of three necrotizing enterocolitis models in premature rats. Mol Med Report 2011;4:1333–1338.

[22] Kosloske AM. Epidemiology of necrotizing enterocolitis. Acta Paediatr Suppl 1994;396:2–7.

[23] Young CM, Kingma SD, Neu J. Ischemia-reperfusion and neonatal intestinal injury. J Pediatr 2011;158(Suppl 2):e25–28.

[24] Leaphart CL, Cavallo J, Gribar SC, Cetin S, Li J, Branca MF, Dubowski TD, et al. A critical role for TLR4 in the pathogenesis of necrotizing enterocolitis by modulating intestinal injury and repair. J Immunol 2007;179:4808–4820.

[25] Mohamed A, Shah PS. Transfusion associated necrotizing enterocolitis: a meta-analysis of observational data. Pediatrics 2012;129:529–540.

[26] Paul DA, Mackley A, Novitsky A, Zhao Y, Brooks A, Locke RG. Increased odds of necrotizing enterocolitis after transfusion of red blood cells in premature infants. Pediatrics 2011;127:635–641.

[27] Singh R, Visintainer PF, Frantz ID 3rd, Shah BL, Meyer KM, Favila SA, et al. Association of necrotizing enterocolitis with anemia and packed red blood cell transfusions in preterm infants. J Perinatol 2011;31:176–182.

[28] El-Dib M, Narang S, Lee E, Massaro AN, Aly H. Red blood cell transfusion, feeding and necrotizing enterocolitis in preterm infants. J Perinatol 2011;31:183–187.

[29] Josephson CD, Wesolowski A, Bao G, Sola-Visner MC, Dudell G, Castillejo MI, et al. Do red cell transfusions increase the risk of necrotizing enterocolitis in premature infants? J Pediatr 2010;157:972–978.

[30] Mally P, Golombek SG, Mishra R, Nigam S, Mohandas K, Depalhma H, et al. Association of necrotizing enterocolitis with elective packed red blood cell transfusions in stable, growing, premature neonates. Am J Perinatol 2006;23:451–458.

[31] Blau J, Calo JM, Dozor D, Sutton M, Alpan G, La Gamma EF. Transfusion-related acute gut injury: necrotizing enterocolitis in very low birth weight neonates after packed red blood cell transfusion. J Pediatr 2011;158:403–409.

[32] Krimmel GA, Baker R, Yanowitz TD. Blood transfusion alters the superior mesenteric artery blood flow velocity response to feeding in premature infants. Am J Perinatol 2009;26:99–105.

[33] Sherman MP. New concepts of microbial translocation in the neonatal intestine: mechanisms and prevention. Clin Perinatol 2010;37:565–579.

[34] Cheu HW, Brown DR, Rowe MI. Breath hydrogen excretion as a screening test for the early diagnosis of necrotizing enterocolitis. Am J Dis Child 1989;143:156–159.

[35] Treszl A, Tulassay T, Vasarhelyi B. Genetic basis for necrotizing enterocolitis: risk factors and their relations to genetic polymorphisms. Front Biosci 2006;11:570–580.

[36] Marques TM, Wall R, Ross RP, Fitzgerald GF, Ryan CA, Stanton C. Programming infant gut microbiota: influence of dietary and environmental factors. Curr Opin Biotechnol 2010;21:149–156.

[37] Schwiertz A, Gruhl B, Löbnitz M, Michel P, Radke M, Blaut M. Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full-term infants. Pediatr Res 2003;54:393–399.

[38] Sántulli TV, Schullinger JN, Heird WC, Gongaware RD, Wigger J, Barlow B, et al. Acute necrotizing enterocolitis in infancy: a review of 64 cases. Pediatrics 1975;55:376–387.

[39] Schullinger JN, Mollitt DL, Vinocur CD, Sántulli TV, Driscoll JM Jr. Neonatal necrotizing enterocolitis: survival, management and complications: a 25-year study. Am J Dis Child 1981;135:612–614.

[40] Grosfeld JL, Cheu H, Schlatter M, West KW, Rescorla FJ. Changing trends in necrotizing enterocolitis. Experience with 302 cases in two decades. Ann Surg 1991;214:300–306.

[41] Yee WH, Soraisham AS, Shah VS, Aziz K, Yoon W, Lee SK; Canadian Neonatal Network. Incidence and timing of presentation of necrotizing enterocolitis in preterm infants. Pediatrics 2012;129:e298–304.

[42] MacKendrick W, Caplan M. Necrotizing enterocolitis: new thoughts about pathogenesis and potential treatment. Pediatr Clin North Am 1993 40:1047–1059.

[43] Musemeche CA, Kosloske AM, Bartow SA, Umland ET. Comparative effects of ischemia, bacteria, and substrate on the pathogenesis of intestinal necrosis. J Pediatr Surg 1986;21:536–538.

[44] Engel RR, Virnig NL, Hunt CE, Levitt MD. Origin of mural gas in necrotizing enterocolitis. Pediatr Res 1973;7:292.

[45] Kosloske AM, Ulrich JA. A bacteriologic basis for the clinical presentations of necrotizing enterocolitis. J Pediatr Surg 1980;15:558–564.

[46] Bucher BT, McDuffie LA, Nurmohammad S, Tarr PI, Warner BB, Hamvas A, et al. Bacterial DNA content in the intestinal wall from infants with necrotizing enterocolitis. J Peditric Surgery 2011;46:1029–1033.

[47] Book LS, Overall JC, Herbst JJ, Britt MR, Epstein B, Jung AL. Clustering of necrotizing enterocolitis: interruption by infection control measures. N Engl J Med 1977;297:984–986.

[48] Bury RG, Tudehope D. Enteral antibiotics for preventing necrotizing enterocolitis in low birthweight or preterm infants. Cochrane Database Syst Rev 2001;1:CD000405.

[49] Palmer SR, Biffin A, Gamsu HR. Outcome of neonatal necrotizing enterocolitis: results of the BAPM/CDSC surveillance study, 1981–84. Arch Dis Child 1989;64:388–394.

[50] Scheifele DW, Olsen E, Fussell S, Pendray M. Spontaneous endotoxinemia in premature infants: correlations with oral feeding and bowel dysfunction. J Pediatr Gastroenterol Nutr 1985;4:67–74.

[51] Pedersen PV, Hansen FH, Halveg AB, Christiansen ED, Justesen T, Høgh P. Necrotising enterocolitis of the newborn – is it gas-gangrene of the bowel? Lancet 1976;2:715–716.

[52] Yale CE, Balish E, Wu JP. The bacterial etiology of pneumatosis cystoides intestinalis. Arch Surg 1974;109:89–94.

[53] Lawrence GW, Lehmann D, Anian G, Coakley CA, Saleu G, Barker MJ, Davis MW. Impact of active immunisation against enteritis necroticans in Papua New Guinea. Lancet 1990;336:1165–1167.

[54] Yu VY, Joseph R, Bajuk B, Orgill A, Astbury J. Necrotizing enerocolitis in very low birthweight infants: a four-year experience. Aust Paediatr J 1984;20:29–33.

[55] Blakey JL, Lubitz L, Campbell NT, Gillam GL, Bishop RF, Barnes GL. Enteric colonization in sporadic neonatal necrotizing enterocolitis. J Pediatr Gastroenterol Nutr 1985;4:591–595.

[56] de la Cochetière MF, Piloquet H, des Robert C, Darmaun D, Glamiche JP, Roze JC. Early intestinal bacterial colonization and necrotizing enterocolitis in premature infants: the putative role of *Clostridium*. Pediatr Res 2004;56:366–370.

[57] Dittmar E, Beyer P, Fischer D, Schäfer V, Schoepe H, Bauer K, et al. Necrotizing enterocolitis of the neonate with *Clostridium perfringens*: diagnosis, clinical course, and role of alpha toxin. Eur J Pediatr 2008;167:891–895.

[58] Gupta S, Morris JG, Panigrahi P, Nataro JP, Glass RI. Endemic necrotizing enterocolitis: lack of association with a specific infectious agent. Pediatr Infect Dis 1994;13:728–734.

[59] Bjornvad CR, Thymann T, Deutz NE, Burrin DG, Jensen SK, Jensen BB, et al. Enteral feeding induces diet-dependent mucosal dysfunction, bacterial proliferation, and necrotizing enterocolitis in preterm pigs on parenteral nutrition. Am J Physiol Gastrointest Liver Physiol 2008;295:G1092–1103.

[60] Lishman AH, Al Jumaili IJ, Elshibly E, Hey E, Record CO. *Clostridium difficile* isolation in neonates in a special care unit. Lack of correlation with necrotizing enterocolitis. Scand J Gastroenterol 1984;19:441–444.

[61] el-Mohandes AE, Keiser JF, Refat M, Jackson BJ. Prevalence and toxigenicity of *Clostridium difficile* isolates in fecal microflora of preterm infants in the intensive care nursery. Biol Neonate 1993;63:225–229.

[62] Alfa MJ, Robson D, Davi M, Bernard K, Van Caeseele P, Harding GK. An outbreak of necrotizing enterocolitis associated with a novel clostridium species in a neonatal intensive care unit. Clin Infect Dis 2002;35:S101–105.

[63] Chang JY, Shin SM, Chun J, Lee JH, Seo JK. Pyrosequencing-based molecular monitoring of the intestinal bacterial colonization in preterm infants. J Pediatr Gastroenterol Nutr 2011;53:512–519.

[64] Noel GJ, Laufer DA, Edelson PJ. Anaerobic bacteremia in a neonatal intensive care unit: an eighteen-year experience. Pediatr Infect Dis J 1988;7:858–862.

[65] Mollitt DL, Tepas JJ, Talbert JL. The role of coagulase-negative *Staphylococcus* in neonatal necrotizing enterocolitis. J Pediatr Surg 1988;23:60–63.

[66] Coates EW, Karlowicz MG, Croitoru DP, Buescher ES. Distinctive distribution of pathogens associated with peritonitis in neonates with focal intestinal perforation compared with necrotizing enterocolitis. Pediatrics 2005;116:241–246.

[67] Scheifele DW, Bjornson GL, Dyer RA, Dimmick JE. Delta-like toxin produced by coagulase-negative staphylococci is associated with neonatal necrotizing enterocolitis. Infect Immun 1987;55:2268–2273.

[68] Scheifele DW, Bjornson GL. Delta toxin activity in coagulase-negative staphylococci from the bowels of neonates. J Clin Microbiol 1988;26:279–282.

[69] Brooks HJ, McConnell MA,, Corbett J, Buchan GS, Fitzpatrick CE, Broadbent RS. Potential prophylactic value of bovine colostrum in necrotizing enterocolitis in neonates: an in vitro study on bacterial attachment, antibody levels and cytokine production. FEMS Immunol Med Microbiol 2006;48:347–354.

[70] McIntosh SM. The role of coagulase negative staphylococcal delta toxin in necrotizing enterocolitis. MSc thesis, University of Otago, Dunedin, New Zealand.

[71] Tegmark K, Morfeldt E, Arvidson S. Regulation of agr-dependent virulence genes in *Staphylococcus aureus* by RNAIII from coagulase-negative staphylococci. J Bacteriol. 1998;180:3181–3186.

[72] Cheung GY, Rigby K, Wang R, Queck SY, Braughton KR, Whitney AR, et al. *Staphylococcus epidermidis* strategies to avoid killing by human neutrophils. PLoS Pathog 2010;6:e1001133.

[73] Overturf GD, Sherman MP, Scheifele DW, Wong LC. Neonatal necrotizing enterocolitis associated with delta toxin-producing methicillin-resistant *Staphylococcus aureus*. Pediatr Infect Dis J 1990;9:88–91.

[74] Otto M. Virulence factors of the coagulase-negative staphylococci. Front Biosci 2004;9:841–863.

[75] Strunk T, Richmond P, Simmer K, Currie A, Levy O, Burgner D. Neonatal immune responses to coagulase-negative staphylococci. Curr Opin Infect Dis 2007;20:370–375.

[76] Eastwick K, Leeming JP, Bennett D, Millar MR. Reservoirs of coagulase negative staphylococci in preterm infants. Arch Dis Child 1996;74:F99–F104.

[77] Wilson SE, Woolley MM. Primary necrotizing enterocolitis in infants. Arch Surg 1969;99:563–566.

[78] Reid WD, Shannon MP. Necrotizing enterocolitis--a medical approach to treatment. Can Med Assoc J 1973;108:573–576.

[79] Frantz ID 3rd, L'heureux P, Engel RR, Hunt CE. Necrotizing enterocolitis. J Pediatr 1975;86:259–263.

[80] Hoy C, Millar MR, MacKay P, Godwin PGR, Langdale V, Levene MI. Quantitative changes in faecal microflora preceding necrotizing enterocolitis in premature neonates. Arch Dis Child 1990;65:1057–1059.

[81] Krediet TG, van Lelyveld N, Vijlbrief DC, Brouwers HA, Kramer WL, Fleer A, et al. Microbiological factors associated with neonatal necrotizing enterocolitis:protective effect of early antibiotic treatment. Acta Paediatr 2003;92:1180–1182.

[82] Keller R, Pedroso MZ, Ritchmann R, Silva RM. Occurrence of virulence-associated properties in *Enterobacter cloacae*. Infect Immun 1998;66:645–649.

[83] Podschun R, Ullmann U. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev 1998;11:589–603.

[84] Hoffmann KM, Deutschmann A, Weitzer C, Joainig M, Zechner E, Högenauer C, et al. Antibiotic-associated hemorrhagic colitis caused by cytotoxin-producing *Klebsiella oxytoca*. Pediatrics 2010;125:e960–963.

[85] Panigrahi P, Gupta S, Gewolb IH, Morris JG Jr. Occurrence of necrotizing enterocolitis may be dependent on patterns of bacterial adherence and intestinal colonization:

studies in Caco-2 tissue culture and weanling rabbit models. Pediatr Res 1994;36:115–121.

[86] Panigrahi P, Bamford P, Horvath K, Morris JG Jr, Gewolb IH. *Escherichia coli* transcytosis in a Caco-2 cell model: implications in neonatal necrotizing enterocolitis. Pediatr Res 1996;40:415–421.

[87] Yan QQ, Condell O, Power K, Butler F, Tall BD, Fanning S. *Cronobacter* species (formerly known as *Enterobacter sakazakii*) in powdered infant formula: a review of our current understanding of the biology of this bacterium. J Appl Microbiol 2012;113:1–15.

[88] Petrosyan M, Guner YS, Williams M, Grishin A, Ford HR. Current concepts regarding the pathogenesis of necrotizing enterocolitis. Pediatr Surg Int 2009;25:309–318.

[89] van Acker J, de Smet F, Muyldermans G, Bougatef A, Naessens A, Lauwers S. Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. J Clin Microbiol 2001;39:293–297.

[90] Stein H, Beck J, Solomon A, Schmaman A. Gastroenteritis with necrotizing enterocolitis in premature babies. Br Med J 1972;2:616–619.

[91] Tzialla C, Civardi E, Borghesi A, Sarasini A, Baldanti F, Stronati M. Emerging viral infections in neonatal intensive care unit. J Matern Fetal Neonatal Med 2011;24 Suppl 1:156–158.

[92] Turcios-Ruiz RM, Axelrod P, St John K, Bullitt E, Donahue J, Robinson N, et al. Outbreak of necrotizing enterocolitis caused by norovirus in a neonatal intensive care unit. J Pediatr 2008;153:339–344.

[93] Bagci S, Eis-Hübinger AM, Franz AR, Bierbaum G, Heep A, Schildgen O, et al. Detection of astrovirus in premature infants with necrotizing enterocolitis. Pediatr Infect Dis J 2008;27:347–350.

[94] Lodha A, de Silva N, Petric M, Moore AM. Human torovirus: a new virus associated with neonatal necrotizing enterocolitis. Acta Paediatr 2005;94:1085–1088.

[95] De Villiers FP, Driessen M. Clinical neonatal rotavirus infection: association with necrotising enterocolitis. S Afr Med J 2012;102:620–624.

[96] Harvala H, Simmonds P. Human parechoviruses: biology, epidemiology and clinical significance. J Clin Virol 2009;45:1-9.

[97] Birenbaum E, Handsher R, Kuint J, Dagan R, Raichman B, Mendelson E, et al. Echovirus type 22 outbreak associated with gastro-intestinal disease in aneonatal intensive care unit. Am J Perinatol 1997;14:469–473.

[98] Rousset S, Moscovici O, Lebon P, Barbet JP, Helardot P, Macé B, et al. Intestinal lesions containing coronavirus-like particles in neonatal necrotizing enterocolitis: an ultrastructural analysis. Pediatrics 1984;73:218–224.

[99] Bhowmick R, Halder UC, Chattopadhyay S, Chanda S, Nandi S, Bagchi P, et al. Rotaviral enterotoxin nonstructural protein 4 targets mitochondria for activation of apoptosis during infection. J Biol Chem 2012 doi:10.1074/jbc.M112.369595.

[100] Dickman KG, Hempson SJ, Anderson J, Lippe S, Zhao L, Burakoff R, et al. Rotavirus alters paracellular permeability and energy metabolism in Caco-2 cells. Am J Physiol Gastrointest Liver Physiol 2000;279:G757–766.

[101] Troeger H, Loddenkemper C, Schneider T, Schreier E, Epple HJ, Zeitz M, et al. Structural and functional changes of the duodenum in human norovirus infection. Gut 2009;58:1070–1077.

[102] Ullrich T, Tang YW, Correa H, Garzon SA, Maheshwari A, Hill M, et al. Absence of gastrointestinal pathogens in ileum tissue resected for necrotizing enterocolitis. Pediatr Infect Dis J 2012;31:413–414.

[103] Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, et al. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe 2007;2:119–129.

[104] Stewart C, Marrs E, Magorrian S, Nelson A, Lanyon C, Perry J, et al. The preterm gut microbiota: changes associated with necrotising enterocolitis and infection. Acta Paediatr 2012;doi: 10.1111/j.1651-2227.2012.02801.x.

[105] Mai V, Young CM, Ukhanova M, Wang X, Sun Y, Casella G, et al. Fecal microbiota in premature infants prior to necrotizing enterocolitis. PLoS One 2011;6:e20647.

[106] Smith B, Bodé S, Petersen BL, Jensen TK, Pipper C, Kloppenborg J, et al. Community analysis of bacteria colonizing intestinal tissue of neonates with necrotizing enterocolitis. BMC Microbiol 2011;11:73.

[107] Wang Y, Hoenig JD, Malin KJ, Qamar S, Petrof EO, Sun J, et al. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. ISME J 2009;3:944–954.

[108] Tanaka S, Kobayashi T, Songjinda P, Tateyama A, Tsubouchi M, Kiyohara C, et al. Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. FEMS Immunol Med Microbiol 2009;56:80–87.

[109] Kenyon S, Boulvain M, Neilson JP. Antibiotics for preterm rupture of membranes. Cochrane Database Syst Rev 2010;8:CD001058.

[110] Cotten CM, Taylor S, Stoll B, Goldberg RN, Hansen NI, Sánchez PJ, et al; NICHD Neonatal Research Network. Prolonged duration of initial empirical antibiotic treatment is associated with increased rates of necrotizing enterocolitis and death for extremely low birth weight infants. Pediatrics 2009;123:58–66.

[111] Millar MR, Linton CJ, Cade A, Glancy D, Hall M, Jalal H. Application of 16S rRNA gene PCR to study bowel flora of preterm infants with and without necrotizing enterocolitis. J Clin Microbiol 1996;34:2506–2510.

[112] Hunter CJ, Upperman JS, Ford HR, Camerini V. Understanding the susceptibility of the premature infant to necrotizing enterocolitis (NEC). Pediatr Res 2008;63:117–123.

[113] Smith B, Bodé S, Skov TH, Mirsepasi H, Greisen G, Krogfelt KA. Investigation of the early intestinal microflora in premature infants with/without necrotizing enterocolitis using two different methods. Pediatr Res 2012;71:115–120.

[114] Are A, Aronsson L, Wang S, Greicius G, Lee YK, Gustafsson J-Å, et al. *Enterococcus faecalis* from newborn babies regulate endogenous PPARg activity and IL-10 levels in colonic epithelial cells. PNAS 2008;105:1943–1948.

[115] Wang S, Ng LH, Chow WL, Lee YK. Infant intestinal *Enterococcus faecalis* down-regulates inflammatory responses in human intestinal cell lines. World J Gastroenterol 2008;14:1067–1076.

[116] Aziz N, Cheng YW, Caughey AB. Neonatal outcomes in the setting of preterm premature rupture of membranes complicated by chorioamnionitis. J Matern Fetal Neonatal Med 2009;22:780–784.

[117] Reiman M, Kujari H, Ekholm E, Lapinleimu H, Lehtonen L, Haataja L, PIPARI Study Group. Interleukin-6 polymorphism is associated with chorioamnionitis and neonatal infections in preterm infants. J Pediatr 2008;153:19–24.

[118] Gonçalves LF, Chaiworapongsa T, Romero R. Intrauterine infection and prematurity. Ment Retard Dev Disabil Res Rev 2002;8:3–13.

[119] DiGiulio DB, Romero R, Kusanovic JP, Gómez R, Kim CJ, Seok KS, et al. Prevalence and diversity of microbes in the amniotic fluid, the fetal inflammatory response, and pregnancy outcome in women with preterm pre-labor rupture of membranes. Am J Reprod Immunol 2010;64:38–57.

[120] Gantert M, Been JV, Gavilanes AW, Garnier Y, Zimmermann LJ, Kramer BW. Chorioamnionitis: a multiorgan disease of the fetus? J Perinatol 2010;30:S21–30.

[121] Been JV, Rours IG, Kornelisse RF, Lima Passos V, Kramer BW, Schneider TA, et al. Histologic chorioamnionitis, fetal involvement, and antenatal steroids: effects on neonatal outcome in preterm infants. Am J Obstet Gynecol 2009;201:587 e1–8.

[122] Elimian A, Verma U, Beneck D, Cipriano R, Visintainer P, Tejani N. Histologic chorioamnionitis, antenatal steroids, and perinatal outcomes. Obstet Gynecol 2000;96:333–336.

[123] Lau J, Magee F, Qiu Z, Houbé J, Von Dadelszen P, Lee SK. Chorioamnionitis with a fetal inflammatory response is associated with higher neonatal mortality, morbidity,

and resource use than chorioamnionitis displaying a maternal inflammatory response only. Am J Obstet Gynecol 2005;193:708–713.

[124] Dempsey E, Chen MF, Kokottis T, Vallerand D, Usher R. Outcome of neonates less than 30 weeks gestation with histologic chorioamnionitis. Am J Perinatol 2005;22:155–159.

[125] Soraisham AS, Singhal N, McMillan DD, Sauve RS, Lee SK; Canadian Neonatal Network. A multicenter study on the clinical outcome of chorioamnionitis in preterm infants. Am J Obstet Gynecol 2009;200:372e1–6.

[126] Been JV, Lievense S, Zimmermann LJ, Kramer BW, Wolfs TG. Chorioamnionitis as a risk factor for necrotizing enterocolitis: a systematic review and meta-analysis. J Pediatr 2012; doi.org/10.1016/j.jpeds.2012.07.012.

[127] Wolfs TG, Buurman WA, Zoer B, Moonen RM, Derikx JP, Thuijls G, et al. Endotoxin induced chorioamnionitis prevents intestinal development during gestation in fetal sheep. PLoS One 2009;4:e5837.

[128] Wolfs TG, Kallapur SG, Polglase GR, Pillow JJ, Nitsos I, Newnham JP, et al. IL-1α mediated chorioamnionitis induces depletion of FoxP3+ cells and ileal inflammation in the ovine fetal gut. PLoS One 2011;6:e18355.

[129] Bersani I, Thomas W, Speer CP. Chorioamnionitis - the good or the evil for neonatal outcome? J Matern Fetal Neonatal Med 2012;25 Suppl 1:12–16.

[130] Hendson L, Russell L, Robertson CM, Liang Y, Chen Y, Abdalla A, et al. Neonatal and neurodevelopmental outcomes of very low birthweight infants with histologic chorioamnionitis. J Pediatr 2011;158:397–402.

[131] Jiménez E, Marín ML, Martín R, Odriozola JM, Olivares M, Xaus J, et al. Is meconium from healthy newborns actually sterile? Res Microbiol 2008;159:187–193.

[132] Blackburn ST. Gastrointestinal and hepatic systems and perinatal nutrition. In: Maternal, Fetal and Neonatal Physiology 2007;447–448 (Saunders Elsevier, St. Louis, Missouri, USA).

[133] Carrion V, Egan EA. Prevention of neonatal necrotizing enterocolitis. J Pediatr Gastroenterol Nutr 1990;11:317–323.

[134] Piena-Spoel M, Albers MJ, ten Kate J, Tibboel D. Intestinal permeability in newborns with necrotising enterocolitis and controls: does the sugar absorption test provide guideline for the time to (re-) introduce enteral nutrition? J Pediatr Surg 2001;36:587–592.

[135] McGuckin MA, Linden SK, Sutton P, Florin TH. Mucin dynamics and enteric pathogens. Nat Rev Microbiol 2011;9:265–278.

[136] Bergstrom KSB, Kissoon-Singh V, Gibson DL, Ma C, Montero M, Sham HP, et al. Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. PLoS Pathog 2010;6:e1000902.

[137] Berg RD. Bacterial translocation from the gastrointestinal tract. Trends Microbiol 1995;3:149–154.

[138] Khailova L, Dvorak K, Arganbright KM, Williams CS, Hapern MD, Dvorak B. Changes in hepatic cell junctions structure during experimental necrotizing enterocolitis: effect of EGF treatment. Pediatr Res 2009;66:140–144.

[139] Khailova L, Mount Patrick SK, Arganbright KM, Halpern MD, Kinouchi T, Dvorak B. *Bifidobacterium bifidum* reduces apoptosis in the intestinal epithelium in necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 2010;299:G1118–1127.

[140] Shiou S-R, Yu Y, Chen S, Ciancio MJ, Petrof EO, Sun J and Claud EC. Erythropoietin protects intestinal epithelial barrier function and lowers the incidence of experimental neonatal necrotizing enterocolitis. J Biol Chem 2011;286:12123–12132.

[141] Han X, Fink MP, Delude RL. Proinflammatory cytokines cause NO-dependent and -independent changes in expression and localization of tight junction proteins in intestinal epithelial cells. Shock 2003;19:229–237.

[142] Sheth P, Seth A, Thangavel M, Basuroy S, Rao RK. Epidermal growth factor prevents acetaldehyde-induced paracellular permeability in Caco-2 cell monolayer. Alcohol Clin Exp Res 2004;28:797–804.

[143] Clark JA, Doelle SM, Halpern MD, Saunders TA, Holubec H, Dvorak K, et al. Intestinal barrier failure during experimental necrotizing enterocolitis: protective effect of EGF treatment. Am J Physiol Gastrointest Liver Physiol 2006;291:G938 –949.

[144] O'Callaghan J, Buttó LF, MacSharry J, Nally K, O'Toole PW 2012. Influence of adhesion and bacteriocin production by *Lactobacillus salivarius* on the intestinal epithelial cell transcriptional response. Appl Environ Microbiol 2012;78:5196–5203.

[145] Sharma R and Tepas JJ 3rd. Microecology, intestinal epithelial barrier and necrotizing enterocolitis. Pediatr Surg Int 2010;26:11–21.

[146] Cilieborg MS, Boye M, Sanglid PT. Bacterial colonization and gut development in preterm neonates. Early Hum Dev 2012;88:S41–S49.

[147] Liu Q, Mittal R, Emami CN, Iversen C, Ford HR, Prasadarao NV. Human Isolates of C*ronobacter sakazakii* bind efficiently to intestinal epithelial cells in vitro to induce monolayer permeability and apoptosis. J Surg Res 2012;176:437–447.

[148] Dai D, Walker WA. Protective nutrients and bacterial colonization in the immature human gut. Adv Pediatr 1999;46:353–382.

[149] Hsueh W, Caplan MS, Qu X-W, Tan X-D, De Plaen IG, Gonzalez-Crussi F. Neonatal necrotizing enterocolitis: clinical considerations and pathogenetic concepts. Pediatr Dev Pathol 2002;6:6–23.

[150] Filipovska-Naumovska E. Study on the aetiology of necrotizing enterocolitis in premature neonates. MSc thesis, University of Otago, Dunedin, New Zealand.

[151] Inder A. Role of bacterial toxins in the aetiology of necrotising enterocolitis. Bachelor of Medical Science thesis, University of Otago, Dunedin, New Zealand.

[152] Hertle R. The family of *Serratia* type pore forming toxins. Curr Protein Pept Sci 2005;6:313–325.

[153] Hoffmann KM, Deutschmann A, Weitzer C, Joainig M, Zechner E, Högenauer C,et al. Antibiotic-associated hemorrhagic colitis caused by cytotoxin-producing *Klebsiella oxytoca*. Pediatrics 2010;125:e960–963.

[154] Neu J, Douglas-Escobar M. Gastrointestinal development: implications for infant feeding. In Nutrition in Pediatrics, ed. Duggan C, Watkins JB and Walker VA, 4th ed. pp. 241–249 (BC Decker Inc., Hamilton, Ontario, Canada).

[155] Sharma R, Young C and Neu J. Molecular modulation of intestinal epithelial barrier: contribution of microbiota. J Biomed Biotechnol 2010;doi:10.1155/2010/305879.

[156] Eberl G. Inducible lymphoid tissues in the adult gut: recapitulation of a fetal developmental pathway? Nat Rev Immunol 2005;5:413–420.

[157] Ford H, Watkins S, Reblock K and Rowe M. The role of inflammatory cytokines and nitric oxide in the pathogenesis of necrotizing enterocolitis. J Pediatr Surg 1997;32:275–282.

[158] Sharma R, Tepas JJ 3rd, Hudak ML, Mollitt DL, Wludyka PS, Teng R-J, et al. Neonatal gut barrier and multiple organ failure: role of endotoxin and proinflammatory cytokines in sepsis and necrotizing enterocolitis. J Pediatr Surg 2007;42:454–461.

[159] Emami CN, Choski N, Wang J, Hunter C, Guner Y, Goth K, et al. Role of interlukin-10 in the pathogenesis of necrotizing enterocolitis. Am J Surg 2012;203:428–435.

[160] Gyurko R, Boustany G, Huang PL, Kantarci A, Van Dyke TE, Genco CA. Mice lacking inducible nitrate oxide synthase demonstrate impared killing of *Porphyromonas gingivalis*. Infect Immun 2003;71:4917–4924.

[161] Nadler EP, Dickinson E, Knisely A, Zhang X-R, Boyle P, Beer-Stolz D, et al. Expression of inducible nitric oxide synthase and interleukin-12 in experimental necrotizing enterocolitis. J Surg Res 2000;92:71–77.

[162] Upperman JS, Camerini V, Lugo B, Yotov I, Sullivan J, Ribin J, Clermont G, et al. Mathermatical modeling in necrotizing enterocolitis - a new look at an ongoing problem. J Pediatr Surg 2007;42:445–453.

[163] Soliman A, Michelson KS, Karahashi H, Lu J, Meng FJ, Qu X, Crother TR, et al. Platelet-activating factor induces TLR4 expression in intestinal epithelial cells: implication for the pathogenesis of necrotizing enterocolitis PLoS One 2010;5:e15044, doi:10.1371/journal.pone.0015044.

[164] Schriffen EJ, Trop M, Schroeder S and Carter EA. Platelet-activating factor induces intestinal necrosis, but not septic shock, in germ-free and specific-pathogen-free rodents. Burns 1991;17:276–278.

[165] Jilling T, Simon D, Lu J, Meng FJ, Li D, Schy R, et al. The roles of bacteria and TLR4 in rat and murine models of necrotizing enterocolitis. J Immunol 2006;177:3273–3282.

[166] Caplan MS, Hedlund E, Adler L, Lickerman M, Hsueh W. The platelet-activating factor receptor antagonist WEB 2170 prevents necrotizing enterocolitis in rats. J Pediatr Gastroenterol Nutr 1997;24:296–301.

[167] Lee TS, and Chau LY. Heme oxygenase-1 mediates the anti-inflammatory effects of interleukin-10 in mice. Nat Med 2002;8:240–246.

[168] Jilling T, Lu J, Caplan MS. Intestinal epithelial cell apoptosis, immunoregulatory molecules, and necrotizing enterocolitis. J Clin Cell Immunol 2012;S3:007. doi 10.4172/2155-9899.

[169] Afrazi A, Sodhi CP, Richardson W, Neal M, Good M, Siggers R, et al. New insights into the pathogenesis and treatment of necrotizing enterocolitis: toll-like receptors and beyond. Pediatr Res 2011;69:183–188.

[170] Gribar SC, Sodhi CP, Richardson WM, Anand RJ, Gittes GK, Branca MF, et al. Reciprocal Expression and signalling of TLR4 and TLR9 in the pathogenesis and treatment of necrotizing enterocolitis. J Immunol 2009;182:636–646.

[171] Le Mandat Schultz A, Bonnard A, Barreau F, Aigrain Y, Pierre-Louis C, Berrebi D, et al. Expression of TLR-2, TLR-4, NOD2 and pNF-κB in a neonatal rat model of necrotizing enterocolitis. PLoS One 2007;2:e1102. doi:10.1371/journal.pone0001102.

[172] Liu Y, Zhu L, Fatheree NY, Liu X, Pacheco SE, Tatevian N, Rhoads JM. Changes in intestinal Toll-like receptors and cytokines precede histological injury in a rat model of necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 2009;297:G442–450.

[173] Arancibia SA, Beltran CJ, Aguirre IM, Silva P, Peralta AL, Malinarich F, et al. Toll-like receptors are key participants in innate immune responses. Biol Res 2007;40:97–112.

[174] Jilling T, Lu J, Jackson M, Caplan MS. Intestinal epithelial apoptosis initiates gross bowel necrosis in an experimental rat model of neonatal necrotizing enterocolitis. Pediatr Res 2004;55:622–629.

[175] Fink SL, Cookson BT. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infect Immun 2005;73:1907–1916.

[176] Hickey L, Jacobs SE, Garland SM. Probiotics in neonatology. J Paediatr Child Health 2012;doi:10.1111/j.1440-1754.2012.02508.x.

[177] Caplan MS, Jilling T. Neonatal necrotizing enterocolitis: possible role of probiotic supplementation. J Pediatr Gastroenterol Nutr 2000;30:S18–22.

[178] Deshpande GC, Rao SC, Keil AD, Patole SK. Evidence-based guidelines for use of probiotics in preterm neonates. BMC Med 2011;9;92.doi: 10.1186/1741-7015-9-92.

[179] Deshpande G, Rao S, Patole S, Bulsara M. Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. Pediatrics 2010;125:921–930.

[180] Morowitz MJ, Poroyko V, Caplan M, Alverdy J, Liu DC. Redefining the role of intestinal microbes in the pathogenesis of necrotizing enterocolitis. Pediatr 2010;125:777–785.

[181] Cario E, Gerken G and Podolsky DK. Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. Gastroenterology 2007;132:1359–1374.