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Mapping the New World of Necrotizing Enterocolitis (NEC): Review and Opinion

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Abstract

A comprehensive review of necrotizing enterocolitis (NEC) is provided; including history, biological basis and frequently asked questions. In addition, a system of improved NEC classification is explained in detail (consisting of five NEC subsets and four NEC-like diseases), to aid the bedside clinician in therapy and prevention. The authors offer opinion for therapeutics in italics at the end of each definition.

Keywords

necrotizing enterocolitis; neonate; premature infant; classification

A BRIEF HISTORY OF NEC

Much of our clinical understanding of NEC comes from the pre-surfactant era. The first reports are from the 1950s (in German), and the first English reports are from the 1960s.[1–4] The use of Bell's staging criteria originates from the 1970s when NEC still predominantly occurred in term and near term infants.[5] Seminal breakthroughs occurred in this decade (including CPAP and total parental nutrition) and with them a "new NEC" emerged in increasingly premature infants that survived because of these technologies. Some suggested that NEC was not a single disease entity, but rather a disease spectrum, perhaps in part because NEC had expanded into these new survivors.[6–8] Some groups documented increased NEC with surfactant introduction, but eventually NEC in older infants declined and the new NEC became static, essentially resetting incidence at a level comparable to the presurfactant era.

The debate about whether or not NEC was a singular disease or a spectrum persisted but the practicality of Bell's staging criteria moved the field towards an inclusive approach and became the *de facto* definition of NEC. A variety of acquired neonatal intestinal diseases (ANIDs) were in-advertently included in NEC cohorts and datasets. However, in the late 1980s, clinicians gradually realized that A) some patients were simply not as sick as the others and B) combining heterogeneous causes of ANIDs into datasets obstructed progress.

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The first modification of the prevailing NEC definition was the exclusion of Bell's stage I (Table 1).[9,10]

In the post surfactant era, a new kind of ANID emerged. Spontaneous intestinal perforation (SIP) was first described in a term infant in 1956 in the *New England Journal of Medicine*, but in the post surfactant era an extremely premature version of it emerged and became rampant during the early postnatal steroid trials.[11–13] It took years for investigators to determine there was a robust relationship between early postnatal steroid therapy and SIP.[14–18] Subsequent research confirmed SIP to be a separate disease entity from NEC.[19] This prompted a rethinking of the prevailing NEC definition (i.e. modified Bell's staging). SIP most commonly presents as pneumo-peritoneum without pneumatosis but was, and in some datasets still is, being miss-classified as Bell's stage IIIb.[20] Because of this, it has been suggested that Bell's staging be abandoned in favor of a new ANID taxonomy.[21] The goal of such a system (Gordon's classifications), was not to stage the severity of disease (other than surgery and mortality), but to focus on the different etiologies of the two known ANIDs at the time of diagnosis. This manuscript furthers this concept, by coupling Gordon's classifications with NEC reductionism (the science of dividing NEC into reproducible subgroups) and provides an up-to-date review of NEC ontogeny.[22]

WHAT IS NEC AND WHAT IS NOT?

Crucial to NEC research is the ability to investigate NEC through databases. In order for this to occur, clinicians and investigators must agree on a common reliable definition of NEC. The challenge faced today is that SIP can be a substantial contaminant of NEC databases if it is not removed. SIP predominantly affects premature infants, whereas NEC affects newborns of all gestational ages although it is more frequent at lower gestational ages (Figure 1).[23–32] Finally, SIP has distinct, non-ischemic histopathology.[23] Cases show thinned or segmental necrosis of the muscularis interna (consistent with mesenteric-specific atrophy) but good mucosal integrity. In contrast, coagulative necrosis of the mucosa with focal hemorrhage is a universal finding in NEC and is presumed to correlate with ischemia (Figure 2).[33] Moreover, animal models demonstrate fundamentally opposing etiologies for SIP versus NEC.[34,35] If we are to understand NEC, SIP must be excluded from NEC datasets.

The differentiation of NEC from SIP at the bedside (first published by Gordon et al.), has been validated to have the best sensitivity and specificity for NEC and should be the modern standard for creating clean NEC datasets (Table 1).[21,23,36] At the time of this publication, The Intermountain Healthcare, and the NICHD databases (although not all individual NICHD sites) have definitions consistent with Gordon's classifications. The Pediatrix Corporation uses diagnostic categories consistent with Gordon's classifications, (but there is no verification of diagnostic accuracy at the level of data entry). The Vermont Oxford Network and the Canadian Neonatal Network have well defined and verified definitions of NEC but these do not effectively exclude SIP (Gordon, personal communication).

SIP is not our only confounder. Mucosal necrosis is a common endpoint, with many etiologies. Equally important to our mission to reduce NEC is the understanding of spurious triggers of mucosal necrosis. NEC, as neonatology has known it for half a century requires three basic pre-conditions: 1) a recently colonized and naïve bowel (i.e. a neonatal bowel), 2) a substantial amount of food in that bowel, 3) a trigger event that compromises the integrity of the mucosal barrier. In recent years we have seen the emergence of entities that we (the authors of this review), describe as "NEC-like diseases" (NLDs). These entities lack one or more of the NEC pre-requisites. This is not to say that such entities are not important or that they should not be tracked. Rather, each entity is associated with a unique risk factor

that has altered the ground rules for NEC. Such risk factors must be identified and understood, but collectively, NLDs obscure our understanding of true NEC and need to be excluded from NEC datasets (we review these later in the manuscript).

WHAT IS THE BIOLOGICAL BASIS OF NEC VULNERABILITY?

When does NEC occur?

A fundamental issue with the newborn bowel is that its immunity is dependent upon the innate system rather than the adaptive until feeding is successfully established. In that developmental interim, toll-like receptors survey the lumen for macromolecules specific to pathogens. If they are activated, they induce apoptosis to entrap the pathogen in the dying enterocyte. Toll-like receptor 4 (TLR4) is especially important, as it senses lipopolysaccharide (LPS).[37,38] TLR4 is known to gradually increase in expression with increasing gestational age, until term birth, when its abundance falls precipitously and its surface expression is actively down regulated (Figure 2). TLR4 expression also increases in response to LPS. The more TLR4 is expressed, the more vigorous the apoptosis and severity of NEC. TLR4 may be the biologic “clock” that sets the timing of NEC onset by an infant’s gestation at birth (illustrated in Figures 1 and 2). This observation may also explain why NEC incidence is highest in the lowest gestation survivors. TLR4 expression is attenuated by breast milk, potentially explaining one of breast milk feedings many benefits.[35]

What determines the severity of NEC?

Mucosal Injury—Several studies indicate that the preterm intestine may have a pro-inflammatory bias, which may be particularly evident in epithelial cells and resident macrophages. Unlike the adult intestine, intestinal epithelial cells (IECs) in the preterm intestine express HLA-I and HLA-DR and could serve as non-professional antigen-presenting cells,[39–44] express a large repertoire of innate response receptors, and can produce a significant local acute inflammatory response. Upon exposure to bacterial products such as lipopolysaccharide (LPS), fetal IECs produce more interleukin (IL)-8 than ileal/colonic epithelial cells from adult subjects.[45–48,35] Fetal IECs can also produce tumor necrosis factor, IL-1, IL-6, and platelet-activating factor (PAF).[49] This inflammatory hyper-responsiveness of fetal IECs correlates with increased nuclear factor kappa B (NF-κB)-mediated gene transcription,[50] which, in turn, is related to a developmental deficiency of the inhibitor of kappa B (IκB).[51] NF-κB signals undergo gradual down regulation in IECs following birth at term gestation.[52]

Bacteria that breach the gut epithelial barrier are normally eliminated by resident macrophages in the *lamina propria*.[53,54] Macrophages first appear in the developing intestine at 11–12 weeks of gestation and increase rapidly during the second trimester.[41,42,55–58] In the adult intestine, macrophages display a profound inflammatory anergy to bacterial products,[54] a unique adaptation that maintains the normal absence of inflammation in the gut mucosa in spite of the close physical proximity to luminal bacteria.[54,59–62] We have shown recently that this inflammatory down regulation of intestinal macrophages is mediated by transforming growth factor (TGF)-β, particularly its isoform TGF-β_2. In the mid-gestation intestine, which is developmentally deficient in TGF-β_2, the non-inflammatory differentiation of gut macrophages is not yet complete. In the event of a preterm delivery, bacterial colonization/translocation before the resident macrophages have undergone specific differentiation may increase the risk of mucosal inflammation and NEC.

Morbidity and Mortality—Some NEC subsets appear to be more severe in their morbidity and mortality than others. For example, transfusion associated NEC has been found to be more severe than other forms of NEC, whereas cow’s milk associated NEC

appears to be less severe.[63] These subsets may originate using mechanisms other than TLR4 as the initial trigger, likely yielding different baselines of severity. In two small single center datasets, NEC with lymphocytosis was found to have high mortality in extremely preterm infants.[30,64] More research is needed to verify and understand these findings.

Why do some infants seem intrinsically vulnerable to NEC?

Although genetic factors are less likely to play a causative role in NEC, epidemiological studies have identified several candidate genes that may modify disease severity and/or outcome. Bhandari *et al.*[65] recorded NEC in either 1 or both of the twins in 9 (14%) of 63 pairs of monozygotic twins and in 29 (15%) of 189 pairs of dizygotic twins. After controlling for covariates, genetic factors did not account for any variance in liability for NEC. However, in other studies, the severity of NEC and its outcome was associated with single nucleotide polymorphisms in several genes, including the IL-4 receptor (+1902G; protective),[66] IL-18 (−607A; increased severity),[68] vascular endothelial growth factor (+450C; increased risk),[69] and carbamoyl-phosphate synthetase 1 (T450N; increased risk).[67] More recently, Sampath *et al.*[70] followed a cohort of 271 preterm infants, where 15 infants (5.6%) developed NEC. They identified the NFKB1 (g.−24519delATTG) variant in all infants with NEC but only in 65% of infants without NEC ($p = 0.003$). In contrast, the NFKBIA (g.−1004A>G) variant was present in 13.3% of infants with NEC but in 49% of infants without NEC (P = 0.007).

How do environmental factors influence the risk of NEC?

There are a variety of environmental factors that increase the risk of NEC (bacterial skew and overgrowth, hypoxia-ischemia, transfusion, cow's milk allergy, and delayed feeding to name the ones that traditional research and NEC reductionism have identified). Platelet activating factor (PAF) is an important acute mediator in the pathway of mucosal necrosis.[71,72] PAF is a soluble phospholipid, generated from existing cell membranes. It is released into the extracellular space by enterocytes in response to a wide variety of metabolic stresses (including hypoxia and inflammation). It is thought to act as an autocrine and paracrine signal, crossing into the synthesizing and adjacent cells then stimulating *de novo* production of TLR4, (thus increasing both the risk and severity of NEC through these local mechanisms of amplification).

What is the role of bacteria in NEC?

Fewer than 15% of blood cultures obtained on patients with NEC are positive.[27] Thus systemic infection is not a common characteristic of NEC. In addition, conventional techniques for stool culture are suboptimal because 80% of the human colonic microbiota is not detected. Recently, taxonomic classification and molecular profiling of microbiota has become possible by sequencing the 16S small subunit bacterial ribosomal RNA gene, allowing identification of previously undetectable microbes.[73] From these studies we've learned that bacteria outnumber human cells 10:1 and their genome out-sizes the human genome by a factor of 100. The microbiome is essential for the normal development of a balanced immune system, but conversely disturbances in immune regulation can modify the microbiome. Although species composition differs greatly between individuals, incredible functional overlap exists and different bacterial types may provide the same metabolic function. Mapping bacterial species diversity with their function is becoming a new way to define the host-microbiome interaction within "enterotypes".[74,75]

Previous studies performed on stool samples have shown an association of NEC with delayed gut colonization after birth, prolonged empiric use of antibiotics, lower microbial diversity, and higher risk of colonization with pathogenic bacteria in the week leading up to the disease.[76–78] However, results of these studies are not consistent and are limited by the

heterogeneity of methods. More importantly, stool samples may not entirely represent the bacteria that exist at the point of injury in the small and large bowel. Bacterial adherence may be important in NEC, as studies in patients with inflammatory bowel diseases have demonstrated increased bacterial attachment to the intestinal epithelium surface compared to controls. In healthy adults, mucosa-associated microbial communities at different sites of the colon are quite distinct from those in stool.[79,80] Similar studies in preterm infants are difficult to perform as intestinal tissue samples can only be obtained from surgical resections rather than endoscopic biopsies. If a microbial signature in stool samples could be reliably used to identify patients at risk for NEC, modern high-throughput technology might allow cost-effective strategies for microbiome screening of NEC. Unfortunately, that technology is not on the immediate horizon due to the complexities described.

To conceptualize the role of enteric bacteria, one must understand how the neonatal bowel senses them. TLR4 senses LPS, but a similar innate immune receptor, TLR9 has antagonistic effects on apoptosis and senses the CpG repeats within the DNA of primarily commensal bacteria.[35] Thus colonization with *lactobacillus* and *bifidobacterium* species reduce the risk of NEC via TLR9 signaling, and down-regulate TLR4 expression. In contrast, PAF senses metabolic stress, up-regulates TLR4 and therefore can indirectly sense bacterial burden. A simplified mental model of the neonatal intestinal innate immune system's ability to sense the commensal versus the total bacterial burden is a teeter-totter. PAF sits on one seat and TRL9 on the other. There is ground under TRL9 and an abyss under PAF. TLR4 sits in the middle and if it loses its balance in the direction of PAF, disease ensues. One other entity may come into play that can sway the balance toward the necrotic abyss. Eosinophils have the capacity to sense N-formyl-methionylleucyl-phenylalanine (fMLP – a bacteria specific metabolite) and may be recruited to the intestine as an additional form of innate defense when there is mucosal barrier dysfunction such as in cow's milk associated (and perhaps transfusion associated) NEC.[81] To complete the analogy, the playground is dismantled when adaptive immunity grows up.

Another fundamental problem for the preterm neonate is early suppression / alteration of intestinal floral with antibiotics. Many preterm infants are treated empirically with antibiotics at birth (and their mothers are treated ante- and perinatally). We know that these antibiotics suppress intestinal flora and that such alterations are associated with an increased risk of NEC.[82,83] There is also compelling evidence that lack of antimicrobial exposure and/or reduced duration of exposure reduces the risk of NEC.[84,85] More recently, antibiotic choice has come into the spotlight. Ampicillin and gentamicin are the most commonly used initial antibiotics. A large before and after retrospective study found a 10-fold reduction in NEC when the participating centers switched to pipericillin-tazobactam as their antibiotic for initial therapy of presumed sepsis.[86] Thus it seems that a substantial portion of NEC might be related to perinatal antibiotic exposure.

Breast milk contains lactobacillus and helps to drive the flora towards normal colonization. Probiotics are live commensal organisms that are introduced by nasogastric tube as a means to colonize the gastrointestinal tract with "normal" flora. Probiotics have been demonstrated to reduce the incidence of NEC, but the FDA has yet to approve these agents for use in neonates and many diverse concerns have been voiced for this reluctance.[87–90] This debate is at times heated, as there are staunch advocates in favor of probiotic use who favor expediated use, versus critics who are pushing for more rigorous trials. The timing, dosing and composition of probiotics have been variable in past studies, making it challenging to find a consensus therapy. Prebiotics are complex sugars added to formulas that have been demonstrated to promote normal flora growth in neonates. These agents are currently available in some commercial term formulas and will likely be available in preterm formulas in the future.

Mother's milk is a natural and inexpensive means to appropriately colonize the neonate that has other benefits not found with probiotics or prebiotics. Mother's milk should be the first choice for driving appropriate intestinal colonization. Pasteurized donor breast milk should be the second choice (because of its prebiotic, bacterial static and anti-inflammatory effects).[91,92]

Why is the preterm intestine more vulnerable to NEC?

As mentioned above, NEC vulnerability in the preterm infant is mediated by many mechanisms related to the transition from innate to adaptive immune function. Although the exact sequence of events and the specific roles and parts of the intestinal immune system require additional clarification, the common pathway includes mucosal barrier disruption with invasion of luminal bacteria secondary to an innate and injurious inflammatory host response (See Figure 2 for Summary Schematic).

Innate Immunity—Over the last decade, most research on NEC pathogenesis has been focused on the first line of defense against invading organisms, the innate arm of the immune system. NEC has been associated with disrupted tight junctions, reduced mucin-producing goblet cells, and fewer Paneth cells.[93–95] However, a large body of work provides compelling evidence that immature signaling through toll-like receptors, in particular the imbalance of signaling between TLR4 and TLR9 is one of the more important drivers of the disease.[35] TLR4 activation increases apoptosis and reduces enterocyte proliferation and migration.[96–98] Enterocyte apoptosis is one of the first histopathologic events in experimental models of NEC[99] and is caused by TLR4 activation in immature small intestinal tissue (but not in immature colonic or adult small intestinal tissues).[96] Specific vulnerability of the preterm infant to NEC may be mediated by failed down-regulation of TLR4 signaling activity, which is characteristic in the gut of older children.[100] TLR4 signaling pathways are continuously up-regulated in intestinal tissues from premature neonates and rats with NEC compared to controls and absence of TLR4 is protective in experimental NEC model.[101–103] Up-regulation of TLR4 in NEC models can be reduced experimentally both by polyunsaturated fatty acids[104] and through activation of TLR9.[37] Novel compounds are in development to specifically suppress TLR4-mediated intestinal inflammation.[38]

Physical separation between bacteria and intestinal epithelial cells is critical for limiting immune activation and maintaining mutual host-bacterial relationship. A 50μm zone of separation composed of a viscous gel-like mucus layer covers the epithelium and inhibits bacterial access to the mucosal surface (Figure 2). This "demilitarized zone" is maintained by Myd88-dependent secretion of bactericidal C-peptide lectin RegIIIγ by intestinal Paneth cells.[105] RegIIIγ limits bacterial colonization of the small intestinal mucosal surface with Gram-positive bacteria and lack of RegIIIγ in mice results in innate immune activation. Especially in the preterm intestine, where mucus production is limited and local defense mechanisms may not be as effective, perturbed spatial relationships between colonizing microbes and host cells is thought to be an important factor in NEC vulnerability.

Adaptive Immunity—The role of T cells in the pathogenesis of NEC has been understudied. This may be explained in part by suboptimal animal models, including the observed attenuated mucosal T cell response in a piglet model of NEC[90] and the delayed ontogeny of T cell responses in mice compared to humans.[106] However, we have known for a long time that T cells are present in the human fetal ileum at early gestation, that they accumulate following chorioamnionitis and they are capable of activation *in vitro*.[107–109] The *in-utero* antigen-challenged preterm infant exhibits precocious lymphoid development and the potential for a vigorous host response upon appropriate antigenic triggers.[110] In

addition to alteration in effector T cell function, there may also be a role for deficient immune regulation in NEC.

To balance the effects of immune activation, the vertebrate immune system employs regulatory cell subsets that protect the host's own tissues from immune-mediated destruction. One major protective subset is $FOXP3^+$ T regulatory cells (Treg), professional immune suppressor cells that are critical in preventing excessive innate and adaptive immune activation in the intestinal tract.[111–115] Patients lacking this subset of regulatory immune cells, due to X-chromosome linked mutations of FOXP3 (IPEX syndrome), exhibit early development of enteropathy, similar to inflammatory bowel disease.[116] Excessive innate immune activation in the preterm intestine can suppress Treg function.[38,117] Treg act through several distinct mechanisms including: a) secretion of inhibitory cytokines (e.g. IL-10, IL-35, TGF-β), b) granzyme and perforin-dependent cytolysis of T effector cells, c) metabolic disruption of T effector cells leading to apoptosis, and d) inhibition of dendritic cell maturation and function.[118]

While functional Treg are generally absent in the newborn mouse, they are abundant in the intestinal mucosa at early gestational ages in preterm infants.[119] Associated with profound local inflammatory signals, patients with surgical NEC exhibit decreased ratios of Treg to effector T cells in the ileum mucosa compared to gestational-age matched non-NEC controls.[120] Although insufficiently studied in clinical trials, nutritional supplements such as Vitamin A metabolites, probiotics or their components can enrich Treg numbers in inflamed regions, induce immune homeostasis and ameliorate experimental colitis.[121–123] The potential to accelerate Treg development exists, and might truncate the NEC window of vulnerability, but more research is needed.

Despite some evidence that enteral immunoglobulin preparations can reduce the risk for NEC[124] and the protective effects of secretory IgA antibodies in human milk, the role and function of B cells in the developing small intestine and a possible relationship to NEC has not been adequately established. An elegant study recently showed that intestinal epithelial cells up-regulate expression of immune and inflammatory genes when the adaptive immune system fails to control the microbiota.[125] The absence of B cells and/or IgA resulted in a functional switch from metabolic to immunologic gene expression resulting in metabolic dysfunction. These studies support a new mechanism for the well-known anti-inflammatory properties of IgA.[126] Treg provide survival signals to lamina propria IgA-producing B cells in part through production of TGF-β and are therefore critical for induction and maintenance of intestinal IgA responses.[127] Thus Treg not only regulate the acute host-response to avoid injury but can also provide long-term immune homeostasis by actively shaping the intestinal microbiome via enhancement of local adaptive immunity and dampened inflammation.[128]

Why does the risk of NEC diminish after infants are post term?

The transition from innate to adaptive immunity appears to be both the moment of greatest risk to the normal preterm infant as well as the finish line toward relative NEC immunity. TLR4 signaling and surface expression decrease as neonates approach term gestation.[37] Around the same time, macrophage activation diminishes.[39] Many of the environmental triggers that have been associated with NEC and one of the NLDs seem to be linked to aberrant intestinal immune phenomenon. These may include transfusion associated NEC and cow's milk associated NEC (both of which are associated with eosinophilia), and IVIG associated NLD, which occurs most commonly in infants with ABO incompatibility. Infants who navigate this window without intestinal immune aberrancy and/or aberrant bacterial growth have a greatly diminished risk of NEC.

NEC REDUCTIONISM

In this section, we describe five subsets of NEC that have been identified in multiple datasets (Figure 3). These entities should not be regarded as discrete, but rather as overlapping triggers along the spectrum of things that cause NEC. This is because patients in datasets have a Heisenberg uncertainty-like quality – they can only be assigned to one subset at a time. At the bedside, with any given case of NEC, more than one etiology may be in play.

Term NEC / Ischemic NEC

NEC in the term / late preterm infant occurs early, relative to premature infants, with the majority presenting by the end of the first week of life.[129–132] Risk factors include formula feeding (versus breast milk), rapid advance of feeding volumes, chorioamnionitis, polycythemia with hyperviscosity, cyanotic heart disease and/or a precedent hypoxic-ischemic event or an illicit maternal drug history (especially cocaine) that leads to mesenteric ischemia. Term NEC is thought to be secondary to events that principally end in mesenteric bowel ischemia. This is presumed to cause acute elevation of PAF and *de novo* TLR4 expression in enterocytes, since hypoxia/ischemia is the principle trigger for NEC in rodent models (which are typically term for their species). There is sufficient feeding in this specific subset to allow heavy bacterial colonization, (but probably not overgrowth – relative to preterm infants (Figure 3)) and this is likely sufficient to activate newly synthesized TLR4 and induce a cascade of apoptosis that progresses to mucosal necrosis. Term NEC has a much lower surgery and mortality rate than preterm NEC.[130] We caution that although term NEC most commonly occurs in older gestation infants, rarely a preterm infant will acquire NEC that is precipitated predominately by mesenteric ischemia (i.e. ischemic NEC). Such infants will not fit with Sartwell's model of timing but will have a precedent severe hypoxic event.

Reasonable preventative measures for the term / ischemic NEC subset are exclusive breast milk feeding and slower feeding advances for infants with precedent hypoxic/ischemic events, congenital heart disease, polycythemia or maternal history of drug abuse. Use of banked breast milk for infants with especially concerning maternal drug abuse histories is an alternative.

NEC Associated with Packed Red Blood Cell Transfusions (NECw/pRBCs)

NECw/pRBCs has been demonstrated to affect preterm infants relatively late in life, consistent with Sartwell's model of disease and in line with preterm NEC in general (Figure 1). It was robustly authenticated in a multicenter retrospective study by Christensen et al. in 2010. Since then more than a dozen publications have appeared, including a meta-analysis summarizing this amazing two years of literature.[133,134] It is one of the most severe forms of NEC in preterm infants with a high rate of surgery and mortality. The timing of NECw/pRBCs is typically within 48 hours of transfusion and commonly within 12 hours.

Recent reports from a center with a two decade history of using O-donor blood demonstrated that blood type AB infants have a higher NEC mortality rate than other blood types (and risk was highest if infants were born to a mother with A or AB blood).[135] A multi-center retrospective report found that patients with NECw/pRBCs had eosinophilia spikes upwards immediately following the transfusions associated with NEC (and an earlier single center study documented neonatal eosinophilia to be most common with transfusion and NEC).[136,137] These findings suggest that AB blood group epitopes, known to be expressed on the neonatal enterocyte,[138] may be vulnerable to serum antibodies in blood products (or perhaps to multiple exposures of low level antibody). Our current favored

hypothesis is that enterocytes become over-whelmed by these passive antibodies, allowing them to opsonize surface antigens, initiate apoptosis and attract eosinophils. A function of eosinophils is to sense opsonized parasites. Eosinophils migrate toward fMLP (a bacterial specific metabolite) and can be recruited to the intestine when there is mucosal barrier dysfunction.[139] Eosinophils release eosinophil granule protein to trigger apoptosis. However, preterm infants in the vulnerable window of Sartwell's model have increased TLR4 expression/signaling, potentially resulting in a synergistic progression to mucosal necrosis. We stress that this is a working hypothesis. No histology from surgical NECw/pRBCs has been published thus far. We will undoubtedly need to refine this model after such studies become available.

One of the most important observations about NECw/pRBCs is that it has variable incidence. Several authors have described the baseline incidence as roughly one third of all preterm NEC cases.[140–142] Two studies evaluating withholding of feeds during transfusions (to reduce the effects of post prandial blood flow) found reduced rates of NEC with this practice.[143,144] Although such data is limited, these findings are encouraging for the prospects of prevention.

Strategies for preventing NECw/pRBCs might include: 1) eliminating or avoiding "late" transfusions by minimizing unnecessary blood draws, 2) use of erythropoiesis stimulating factors and hematinics to avoid transfusion, 3) typing and washing all pRBCs; 4) withholding feedings during transfusions; 5) switching to continuous feedings when a transfusion may be needed soon, and 6) standardizing transfusion criteria (more research in the area of standardization is needed and should include NIRS and Doppler ultrasound of mesenteric artery flow).

NEC Associated with Cow's Milk Intolerance

There is limited data on this entity beyond published case reports.[145–148] The most useful of the case reports show that neonatal disease is generally not associated with robust IGE hypersensitivity but rather the eosinophils are being recruited to the bowel by interleukin-5 elevation.[148] Challenge with cow's milk antigen causes them to release eosinophil granule protein.[149] Pneumatosis intestinalis occurs principally in premature infants. Within the Pediatrix dataset, a search for infants with NEC who demonstrated eosinophilia at the time of presentation yielded a subset of infants with lower mortality, suggesting a more benign form of NEC.[145] Unfortunately no feeding data was available. A follow up study in the Intermountain Healthcare dataset was more fruitful.[130] Neonates with bloody stools at presentation were evaluated for eosinophilia, associated risk factors and outcomes. One subgroup was NECw/pRBCs (who had severe outcomes), but the other half was a group with feeding difficulty, high formula and fortifier exposure but relatively benign outcomes. A little less than half of these infants developed pneumatosis. This would be the precise demographic for cow's milk intolerance evolving into NEC. Switching to elemental formula was found to be curative in all cases where it was tried.

One of the more interesting findings in the Intermountain Healthcare dataset was mild, indolent eosinophilia with cow's milk associated NEC.[130] In older infants, the hallmark of cow's milk allergy is eosinophils in the stool. Our hypothesis is that neonatal cow's milk associated NEC is on the same spectrum, except that they have not yet acquired the full capacity to develop true allergy and so they smolder and trigger intermittent episodes of mucosal necrosis when their intestines are over-challenged with excess antigen. Like NECw/pRBCs, we hypothesize that eosinophils are a crucial component in NEC associated with cow's milk intolerance, only with this disease subset, the immune aberrancy has been actively mediated by the host rather than passively acquired by transfusion.

Prevention of this entity entails exclusive use of human breast milk, introduction of fortifiers after reaching the minimum volume for full feeds (120 mls/kg/day); and when appropriate, use of human based fortifiers. NICUs with higher Caucasian and Oriental prevalence will likely have a higher incidence of this NEC subset based on the racial prevalence of eosinophilia noted in the Pediatrix dataset and the fact that cow's milk allergy is more common in Caucasians and Orientals.[128] *Therapy involves exclusive use of elemental formula, because mother's milk can have sufficient dietary cow antigen to sustain the allergic response once they have become symptomatic.*

NEC Associated with Contagion and/or Lymphocytosis

NEC is known to be seasonal.[149,150] The timing of peak incidence varies from region to region but generally is higher in the Winter and early Spring, coincident with community enteral viruses associated with NEC in case reports and series.[151–162] However, there are very few dataset-based studies with good quality showing the prevalence of virus-associated NEC other than rotavirus.[163] Rough estimates from the seasonality data would place the impact of virus-suspected NEC cases at about ten to fifteen percent above baseline of all NEC.

Another line of evidence that has been emerging more recently is that of lymphocytosis on the CBC at time of NEC diagnosis. Recent single center studies have demonstrated much higher total estimates.[30,64] If the percent lymphocyte count is used (>40%), then 61% of all NEC cases in that institution might be associated with viruses.[64] When the absolute lymphocyte count was used in another single center study, 25% were thought to be potentially viral in nature.[30] In both studies a high lymphocyte count was associated with increased mortality in preterm infants. Infants who have lymphocytosis associated NEC are most often preterm and typically acquire NEC consistent with Sartwell's model. This observation suggests that the lymphocytosis may be more about the timing than the pathogen (if indeed there is one in each case). One of the fundamental difficulties with this research is that it has been unclear what "normal" lymphocyte numbers should be for preterm infants at any given gestation or at any given point of time during their life in the NICU. That should change with a recent publication from the Intermountain Healthcare System, which provides reference ranges for lymphocytes on CBCs by gestation and age of postnatal life.[164]

Prevention of NEC acquired during contagion entails the usual infectious disease strategies employed to prevent spread to others: quarantine of infected infants (preferably in an isolation room), hand washing, glove and gowning, sanitary disposal of diapers in a separate area from other patients, dedicated rather than shared nursing, and visitation policies that address hygiene and communicability of potential pathogens. In addition, bowel decompression and prophylactic antibiotics to suppress bowel flora should also be considered. Some units have begun employing these strategies as preventive measures when infants present with lymphocytosis and ileus. These measures seem logical to us.

NEC Associated with Extreme Prematurity and Delayed Feeding

Perhaps the most challenging NEC subset to define is that of the preterm infant with delayed feeding / feeding intolerance. We know that this entity exists, because it keeps showing up as the NEC control group in surgical NEC or our fulminant NEC cohort studies[165,166] (where it is the majority of cases), but no specific definition exists. Recently, a well powered analysis of nutrition practices demonstrated that early nutrition optimization (including early enteral feeding), decreased the risk of NEC.[167] Biologically speaking, Douglas Burrin's lab established that total parental nutrition causes immediate and lasting mucosal hypoplasia in the absence of enteral feeding.[168] In contrast, tiny amounts of enteral feeding can stimulate

mucosal growth and elemental diets with no biological components are sufficient to do so.[169,170] Mucosal restitution (the process of mucosal renewal through cell proliferation), is dependent upon establishing successful enteral feeding. If restitution becomes imbalanced, mucosal barrier function fails and NEC ensues. Anecdotally, and based on limited retrospective data, this form of NEC may present at lower feeding volumes than other subsets.

Like all other forms of true NEC, this subset is hypothesized to present at a time that is consistent with the Sartwell's model, however data confirming this postulate is not robust and needs to be investigated further. Another weakness of this subset is that there may be substantial overlap with the other forms of preterm NEC, since its triggers have yet to be characterized.

Early introduction of breast milk with a gradual standardized advance is the best current feeding practice for prevention of NEC in the preterm infant and likely has the greatest impact on NEC associated with delayed feeding.

NEC-Like Diseases (NLDs)

In defining NLDs the first tenet is to understand why they are not "true" NEC. If one recalls the three preconditions of NEC (a naïve bowel, food in the bowel and a trigger event), these criteria are relatively simple to satisfy. We offer one additional requirement, namely that true NEC behaves according to Sartwell's model of disease onset. This dramatically improves the rigor of such a definition, allowing us to remove entities that are different because some event or agent has been introduced to the patient that has changed their baseline vulnerability.

NLD Associated with IVIG Administration for Red Blood Cell Hemolysis—There are three reports with a total of 16 cases of IVIG associated NLD in the literature.[171–173] Eleven of these received the IVIG for ABO incompatibility whereas five had rH disease. None of those with rH disease required surgery but seven of the ABO cases did and one of them died. Although a small sample, this is a significant difference ($p < 0.05$) in morbidity by blood type. It seems likely that IVIG associated NLD might be similar in nature to NECw/pRBCs. One important difference is: infants with IVIG associated NLD were often not fed or minimally fed. In other words, they failed to meet the feeding criteria for NEC. Another important difference is that they occur too early to be consistent with Sartwell's model. We postulate that this disease may represent the extreme example of opsonization effect, using anti-AB immunoglobulin in the IVIG to opsonize AB blood group type antigens expressed on the enterocytes with sufficient vigor to induce apoptosis, necrosis and pneumatosis without first acquiring substantial enteral flora. We do not know if these infants had eosinophilia.

A sensible strategy for reducing NLD associated with IVIG is to abstain from IVIG use in infants with ABO disease (which can usually be managed without it).

NLD Associated with Use of Simply Thick—In July of 2011, the FDA issued a warning regarding Simply Thick™ following 15 cases of NEC that had been reported to them.[174] Shortly thereafter they closed one of the three plants that make the product. That plant was closed due to lack of adequate sterility. Thus far the only description of this entity in the literature is a case series from Wake Forrest.[175] The report describes three preterm infants with colonic NEC acquired well after the period predicted by Sartwell's model (either at discharge or post discharge at home). The infants were all on Simply Thick™ for reflux. Simply Thick™ is made from natural gum, which is nearly impossible to digest in the absence of endogenous bacteria growing in the thickening product itself. If gum is

fermented into injurious short chain fatty acids then absorbed directly across the colonic mucosa, a colonic version of NEC may ensue in infants who would otherwise be out of the window of susceptibility.

We recommend that no natural gum thickeners be used in the neonatal population, since it is always possible that a company utilizing natural gum could have a failure in the sterility of its processing. Synthetic thickeners should not have this risk.

NLD Associated with Gastroschisis—Gastroschisis is an abdominal wall birth defect that results in prolonged delays in feeding due to bowel dysmotility. Dated reports estimate the incidence of "NEC" in gastroschisis infants to be 10 times that of their gestational contemporaries.[176,177] Anecdotal experience with gastroschisis indicates that it does not adhere to Sartwell's model and that infants have often been on full feeds for extended periods before they manifest the one ileus episode that yields pneumatosis. Many infants with gastroschisis are NPO for prolonged periods, likely leading to significant mucosal atrophy. In trying to understand this association, we believe that chronic dysmotility increases floral skew and overgrowth, subsequently resulting in NEC. Little is known about the maturation of adaptive immunity in infants with gastroschisis (it is possible that they do not mature as other infants do because of the prolonged exposure of the bowel to amniotic fluid). Clearly more research is needed, but "NEC" associated with gastroschisis should be considered an NLD on the basis of a common congenital defect that is associated with exponential risk (for gestation) and discontinuity with Sartwell's model.

At this time, we do not have any unique insight for the prevention of NLD associated with gastroschisis. An awareness of heightened risk may be sufficient to help clinicians reduce its incidence until better data is available.

Typhlitis—Few people would categorize typhlitis (neutropenic enterocolitis) as NEC, but if one considers this carefully, it has many of the same properties (including food in the bowel, a trigger event and the finding of pneumatosis). Typhlitis is not NEC because the bowel is not naïve (or neonatal) and it does not fit with Sartwell's model. The bowel has been reconditioned to the innate immune state by chemotherapy. Not surprisingly, it occurs most commonly in patients with leukemia and lymphoma, in whom the adaptive immune system is specifically damaged.[178,179] We include typhlitis as a NLD so that our classification system will be complete, but offer no opinion on its management.

WHAT IS THE FUTURE OF NEC RESEARCH?

By classifying NEC by its risk factors and triggers, five subsets can be established. The evidence is stronger for some than others and much work is needed, but this reorganization maps definitive strategies for NEC prevention. Hopefully one day NEC will be like central line infections. Each unit will count the numbers of days, if not the months and years, between NEC cases. For some it is already happening.[180,181] Such institutions generally have the benefit of having mostly inborn patients and thus full control of clinical support decisions. It was once like this with central line infections, until we developed universal definitions and NICUs were able to compare infection data with transparency.[182] NEC reductionism will set and then reset new research priorities as we systematically work to minimize the incidence within the individual NEC subsets. Basic research will continue to refine our understanding of NEC subsets and further validate them. In the past the NICHD has held think tanks on NEC research at pivotal junctures (and some of the authors of this review have been participants).[183,184] Perhaps it is time to do so again.

Surgical NEC, adjusted for inflation since the time of the original publication,[185] is now estimated to add an additional $234,603 per patient and 60 additional hospital days (with 41% mortality). Medical NEC adds an additional $92,858 per patient and 22 additional hospital days (with 22% mortality). In a single year the VON documented nearly 5000 cases of NEC in its NICUs in the United States.[186] The Pediatrix Corporation documented over 10,000 cases in the United States over 12 years in all NICU admits.[29] No one knows how many cases of NEC there are a year in the United States, but likely it is above 10,000. If true, we are spending more than $1.5 billion dollars a year on NEC with hospital costs alone. Much of this is borne by the American taxpayer through the Medicaid program. We must re-engage in a concerted national effort to reduce this impact.

PUTTING ALL THIS INFORMATION TOGETHER AT THE BEDSIDE

One of the challenges of a review of this magnitude is information overload and discerning key points to take to the bedside. The first message is that NEC reductionism improves one's ability to predict the types and timing of triggers that precipitate NEC. The second is that preterm NEC almost always involves multiple phenomena. For example, imagine a 24 week gestation infant who is now corrected to 32 4/7 weeks, stable on room air and tolerating full volume bolus feeds of formula, who suddenly re-develops apnea and has a non-specific distal ileus. The infant has a lymphocytosis of 53% on the CBC and a hematocrit of 24.4%. This patient actually has five concurrent risk factors for NEC: 1) this patient fits the timing of Sartwell's model for disease onset, 2) The baby is anemic and this may or may not be related to the apnea. If one's first act is to transfuse, this may increase the risk of NEC (especially if the infant's blood type is AB and it has already had multiple transfusions that were each associated with transient eosinophilia). 3) the patient is being bolus fed, which creates postprandial blood flow effects (and thus relative mesenteric ischemia), 4) the infant has a lymphocytosis, and an ileus, consistent with an enteral-viral infectious trigger, 5) the infant is not on breast milk as its primary nutrition source and thus does not have many of its NEC protective effects. Reasonable interventional strategies in such an infant might include: stopping feeds, a 48 hour rule out with antibiotics to suppress luminal flora (or longer if pneumatosis becomes evident on x-ray) and quarantine in isolation. Delay or avoidance of transfusion by return to nasal cannula support for apnea might be one way to deal with anemia/apnea (versus typed and matched transfusion while NPO). The decision to use a Replogle tube would depend upon the extent of the ileus. If it appears more obstructive in nature than global, glycerin might be a reasonable alternative. The point we are trying to make is that the support strategies chosen should best address as many of the underlying NEC risk factors as possible. Individual clinicians will undoubtedly choose a variety of interventions, but this may not matter if they are addressing the right issues. The addition of Sartwell's model and NEC reductionism to our clinical lexicon shifts the focus from just clinical support of the immediately apparent clinical issues - to support integrated with NEC prevention.

Clinicians who are cognizant of the multifactorial risks for NEC may reduce its morbidity and mortality in their units. At Tulane-Lakeside Hospital in New Orleans (a level III regional NICU), where the concept of NEC reductionism has been employed for more than two and half years, there has not been a single death or bowel resection associated with NEC since 2009 (total patients admitted = 604 and counting, with no true NEC, 3 cases of SIP and 2 cases of NLD associated with gastroschisis).[181] Anecdotes are not data, but the reader deserves to know if the architect of NEC reductionism can generate meritorious outcomes with it.

CONCLUSION

The separation of NEC from SIP (Gordon's classification) and the subsequent reduction of NEC into subgroups (NEC reductionism) together represent an improved operational framework for more accurately assessing NEC incidence and origin. Basic science has better elucidated the biologic basis of NEC vulnerability and the application of Sartwell's model has helped translate these findings, such that we now better understand when infants are at greatest risk to acquire NEC during their hospital course. The time is now to bring these advances to the bedside. When a clinician can accurately identify and understand NEC, they can work to prevent it.

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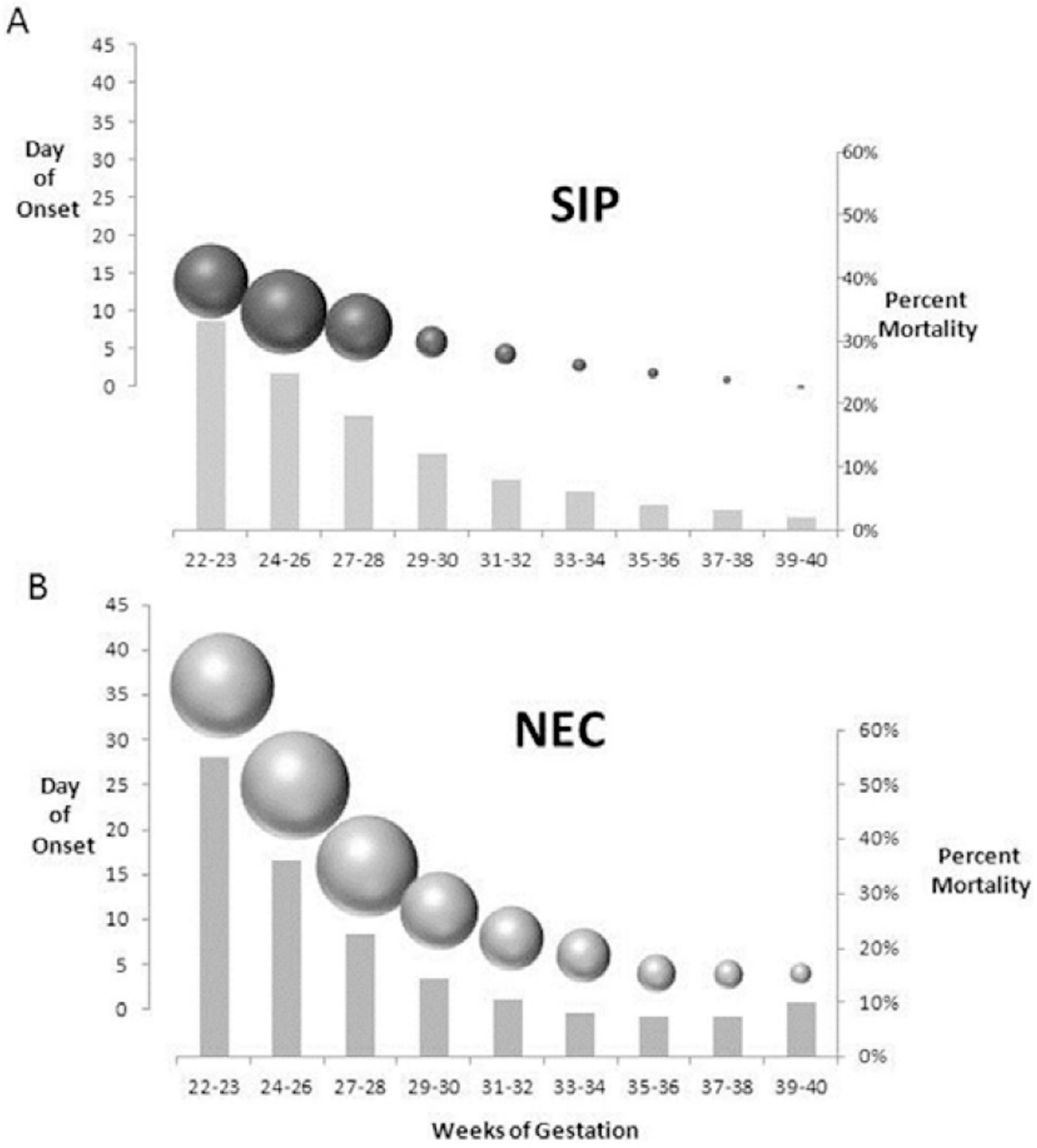

Figure 1.

Summation / consolidation of data about SIP and NEC, including time of presentation, percent mortality and incidence of disease stratified by gestational age. **A:** SIP, the left-sided y-axis equals day of onset indicated by point at the sphere centers. The sphere diameters indicate the percentage of the population of SIP who presented on that day of life (presented as estimated means based on multiple studies). The spheres are arrayed along the x-axis in the underlying graph to show the approximated gestational distribution. The percent mortality stratified by gestational age is indicated by the bars with the values indicated on the right-sided y-axis. **B:** NEC, left-sided y-axis equals day of onset indicated by the sphere

centers. The sphere diameters indicate the percent of the population of NEC that presented on that day of life (mean for the gestational age group), and correlate with the x-axis below. The gray columns indicate the percent mortality (right side y-axis). On average, NEC can be seen to occur later in life at all gestational ages than SIP. NEC also has a higher mortality when compared across gestational strata. It is apparent in this comparison of SIP and NEC, that the timing of presentation, incidence and trends in mortality are different across gestational strata. Data for the extrapolations these figures are based upon are summarized data from references 24–29. In cases where precise data was unavailable in the units needed (example, if the data was available by birth weight instead of gestational age), the data was extrapolated using the assumption that the population as a whole was appropriate for gestational age and the datasets were representative of the same disease entity. For example, in the case where pooled averages of two birth weight groups were the only data available for timing and incidence from a national dataset, gestational spread of a single center report was used to re-apportion the values into the gestational distribution shown (SIP). Likewise, two single center reports were used to overlay the timing of NEC onset onto the national dataset of incidence and mortality.

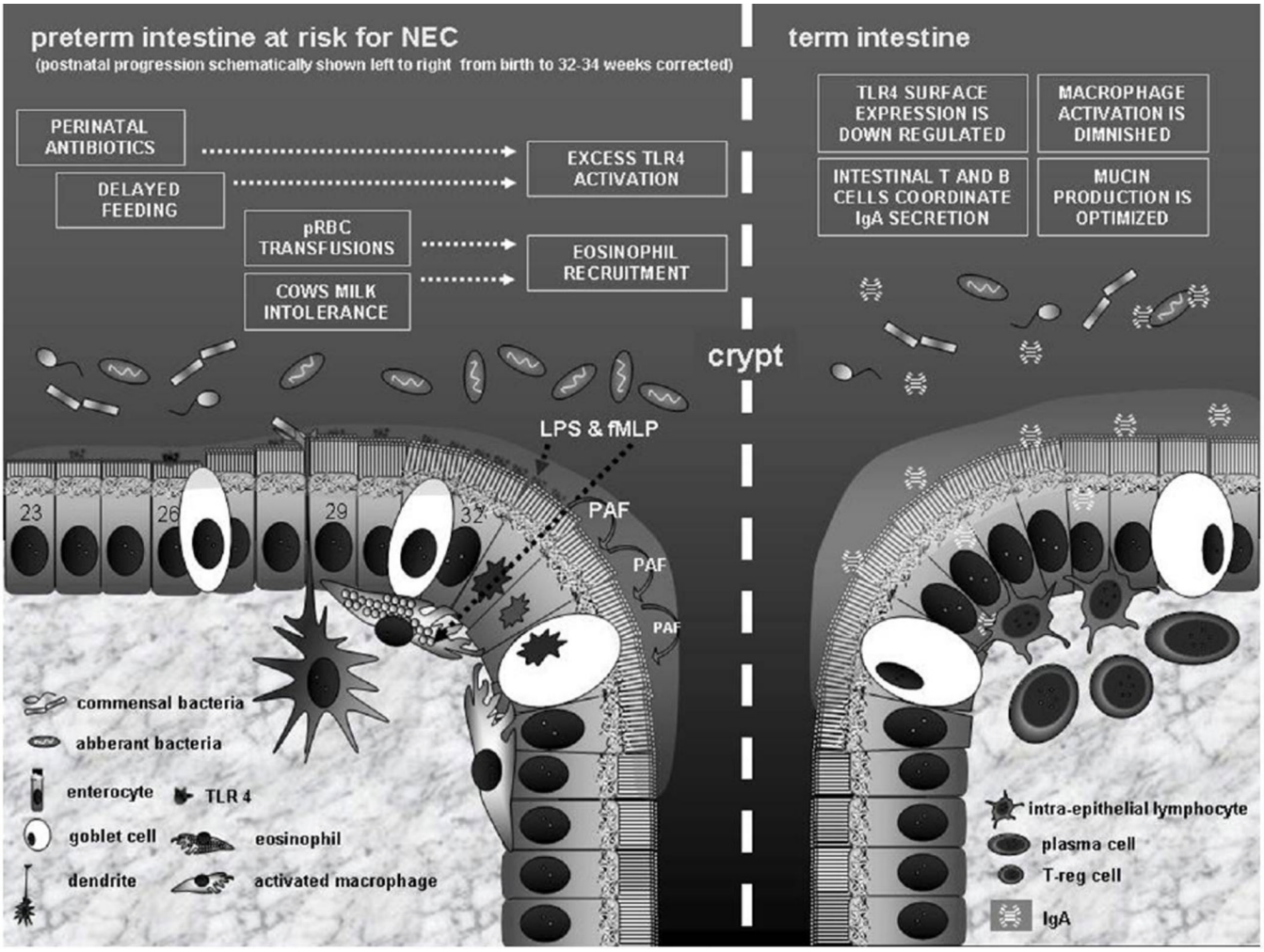

Figure 2.

Schematic illustrating proposed differences in NEC risk between preterm and term intestine at the cellular level of the mucosa. At the top of the schematic, risk modifiers and their resultant effectors are shown in boxes. Immediately above the mucosa, bacteria are shown in appropriate abundance and in relation to the boxed narrative above and the corrected gestation time line below (indicated by the numbers in the intestinal epithelial cells). Innate immune system effectors of cellular apoptosis / necrosis can be seen at or after 32 weeks corrected gestation, consistent with Sartwell's model of incubation. In the term intestine, adaptive immune constituents abrogate NEC risk via active modulation of lumenal flora and down regulation of the innate immune system. ***Abbreviations / legend:*** TLR4 (toll like receptor-4 – principle mediator of enterocyte apoptosis in NEC genesis), fMLP (a bacterial metabolite that acts as a chemoattractant for eosinophils), T reg cells (T regulatory cells), PAF (platelet activating factor), LPS (lipopolysacharide – bacterial metabolite that activates TLR4, pRBC (packed red blood cells).

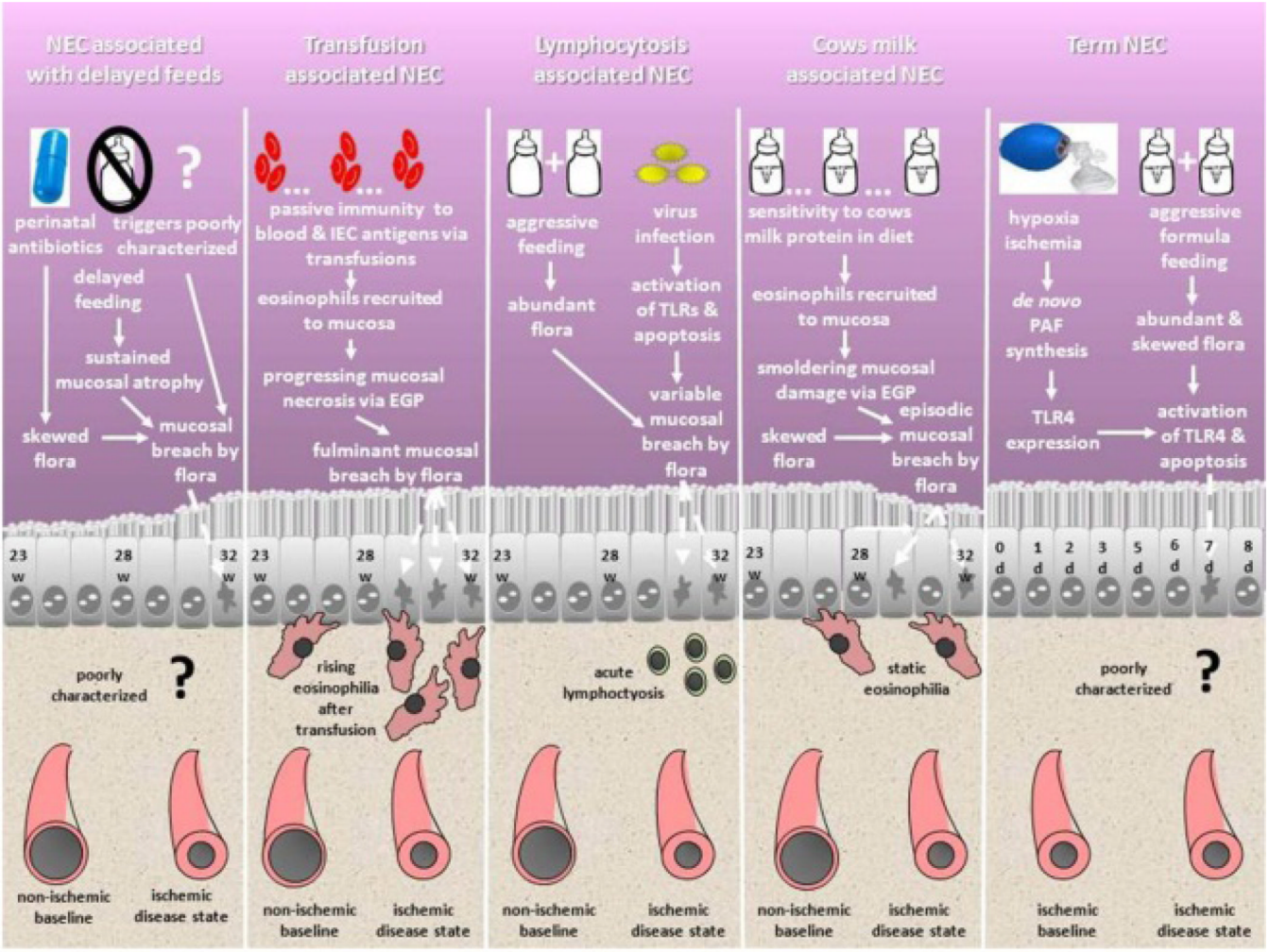

Figure 3.

Schematic of NEC subset ontogeny. Five NEC subsets are shown. At the top of the chart, clinical risk factors and pathways of ontogeny are described. Within the intestinal epithelial cell layers, most common time windows of presentation and mucosal health are characterized. In the bottom portion of the schematic, characteristic findings at presentation on the CBC and the intrinsic vascular tone within the lamina propria of the villi are characterized. Each subset has a novel ontogeny in comparison to the others. NEC after delayed feeds may ultimately be found to be a subcomponent of the other subsets (particularly transfusion and lymphocytosis associated NEC subsets), since rigorous investigation of potential triggers at the time of presentation have not been reported. Term NEC can also rightly be called “ischemia-triggered NEC” and can rarely occur in preterm / late preterm infants when the described precedent conditions are fulfilled. EGP = eosinophil granule protein.

Table 1

Comparison of NEC/ANID Classification System and Their Purposes

Bell's Original Classification of NEC	**Modified Bell's Classification of NEC**	**NEC vs SIP Differentiation**	**NEC Reductionism (NEC Subsets)**
Created to estimate mucosal to serosal progression of disease	**Stage I was dropped to reduce inclusion of non-specific ileus**	**(Gordon's Classification) The only staging is medical vs surgical**	**This expansion takes NEC from ANIDs & reduces it into clinical subsets**
Stage I = ileus		NEC = pneumatosis and/or portal air	- Term NEC - Transfusion-associated NEC
Stage II = pneumatosis	Stage II = pneumatosis	SIP = pneumoperitoneum without pneumatosis	- Cow's milk associated NEC
Stage IIIa = pneumatosis + systemic illness	Stage IIIa = pneumatosis + systemic illness	When in doubt, time of onset should be considered (SIP vs NEC)	- Preterm NEC associated with delayed feeding (not otherwise assigned)
Stage IIIb = Stage IIIa + intestinal perforation	Stage IIIb = Stage IIIa + intestinal perforation	Surgical or pathology findings can overturn clinical diagnoses	- NEC associated with contagion or lymphocytosis - NEC-like diseases (NLD)
Focus was on clinical staging of NEC	**Focus on datasets that contain NEC vs ileus due to sepsis**	**Focus is on getting clean NEC data**	**Focus is on identifying risk factors to facilitate quality improvement & prevention**