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Toward improving mucosal barrier defenses: rhG-CSF plus IgG antibody

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A B S T R A C T

Epithelial cell functions ultimately define the ability of the extremely low birth weight human fetus to survive outside of the uterus. These specialized epithelial cell capacities manage all human interactions with the ex utero world including: i) lung mechanics, surface chemistry and gas exchange, ii) renal tubular balance of fluid and electrolytes, iii) barrier functions of the intestine and skin for keeping bacteria out and water in, plus enabling intestinal digestion, as well as iv) maintaining an intact neuroepithelium lining of the ventricles of the brain and retina. The gut barrier is a clinically relevant model system for studying the complex interplay between innate and adaptive immunity, dendritic and epithelial cell interactions, intraepithelial lymphocytes, M-cells, as well as the gut associated lymphoid tissues where colonization after birth, clinician feeding practices, use of antibiotics as well as exposure to prebiotics, probiotics and maternal vaginal flora all program the neonate for a life-time of immune competence distinguishing "self" from foreign antigens. These barrier defense capacities become destructive during disease processes like necrotizing enterocolitis (NEC) when an otherwise maturationally normal, yet dysregulated and immature immune defense system is associated with high levels of certain inflammatory mediators like TNFα. rhG-CSF has theoretical advantages in managing NEC or sepsis by augmenting neonatal neutrophil number, neutrophil expression of Fcγ and complement receptors, as well as phagocytic function and oxidative burst. rhG-CSF also has potent anti-TNFα functions that may serve to limit extension of tissue destruction while not impairing bacterial killing capacity. Healthy, non-infected neutropenic and septic neonates differ in their ability to respond to rhG-CSF; however, no neonatal clinical trials to date have identified a clear clinical benefit of rhG-CSF therapy. This manuscript will review the literature and evidence available for identifying the ideal subject for cytokine treatment using NEC as the model disease target.

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Epithelial cells define the success of barrier functions

Epithelial cell functions ultimately define the ability of the extremely low birth weight human fetus to survive outside of the uterus. These specialized epithelial cell capacities manage all human interactions with the ex utero world including: i) lung mechanics, surface chemistry and gas exchange, ii) renal tubular balance of fluid and electrolytes, iii) barrier functions of the intestine and skin for keeping bacteria out and water in, plus enabling intestinal digestion, as well as iv) maintaining an intact neuroepithelium lining of the ventricles of the brain and retina.

The mucosal epithelial barrier and gut associated lymphoid tissue work conjointly to allow digestion while simultaneously enabling exclusion of undesirable molecules or organisms. In turn, the normal commensal gut flora assist in digestion, provide vitamin K and aid in maturation of brush border epithelial cell functions.[1-8] When injured, whatever luminal bacteria are present will pass the brush border barrier. If those microorganisms overwhelm local immune defenses, they migrate through the portal vein (~75% of the bowel's vascular drainage) where the organisms would encounter Kupffer cells (tissue macrophages) of the liver as the next level of defense. In turn, Kupffer cells release proinflammatory mediators (e.g. TNF-α and other cytokines) in an enterohepatic loop increasing the propensity for additional mucosal damage.[9-17]

Breaching these mechanisms results in lung tissue macrophages and systemic innate and adaptive immune mechanisms being invoked in order to contain the intrusion. Ultimately, systemic illness engages

the entire body's capacity to muster an immunologic cellular and humoral defense characterized by clinician's at the bedside as septic shock and the multiorgan failure-systemic inflammatory response syndrome.[18-20] Developing a treatment that would contain the infection in the first place and then limit the destructive capacity of invading bacteria in the second place would be ideal.

G-CSF has unexpected and paradoxical advantages

rhG-CSF has Anti-TNF·α and ischemic injury-protective properties

G-CSF (granulocyte colony stimulating factor) is a protein growth factor that increases differentiation of bone marrow progenitors into the neutrophil-type phagocytic lineage. In treated neonates, rhG-CSF (recombinant human G-CSF) increases the absolute neutrophil count (ANC), increases the density of both complement and Fcγ receptors per neutrophil, increases opsonization, phagocytosis, and neutrophil oxidative burst.[21-29] Curiously, rhG-CSF also lowers TNFα levels in critically ill adult humans, neonates and in animal models of sepsis.[25,30-33] rhG-CSF has these effects even in uninfected neutropenic and neutropenic-septic patients but to a lesser extent than in age-matched, uninfected or non-neutropenic human neonatal subjects.[21-24,28-31,34,35] rhG-CSF can also influence a wide array of cytokines and cell responses depending upon timing of its use, under which clinical setting it is applied, as well as the severity of illness.[26,36-41]

In recent animal studies, rhG-CSF facilitated myocardial regeneration, improved revascularization in infarcted hearts and inhibited apoptotic cell death for cardiomyocytes and endothelial cells.[42] rhG-CSF showed neuroprotective effects in animal models of brain ischemia[43] and in Parkinson's disease as well.[44]

Taken together, the combination of bacterial killing, its anti-TNFα actions and the unexpected salutary effects during ischemic injury, suggest that rhG-CSF may have unanticipated benefits in conditions where a systemic inflammatory response-multiorgan failure syndrome exists (Table 1).[9,18-20,26]

Time-dependence of rhG-CSF effects

Host factors. Bone marrow cellular responses to rhG-CSF begin as early as a few hours after intervention causing an egress of near-mature neutrophil progenitors (promyelocytes, metamyelocytes, myelocytes, and bands) into the peripheral blood. This is followed by an acceleration of stem cell entry into the neutrophil differentiation pathway resulting in a subsequent egress of more phagocytic cells after 3-4 days showing a peak effect in neonates at 10-14 days following the initiation of a 3 day course of treatment (likely reflecting stoichiometric entry of stem cells into this lineage).[22,27,36,45] Thus, if a patient were to benefit from intervention, their disease process must itself persist beyond this stage and the patient must be able to survive long enough using conventional support until any putative rhG-CSF effects have sufficient time to occur. In other words, interceding at an inappropriate time has the potential to obfuscate any potential benefits – a likely problem in several studies (Figure 1).[36]

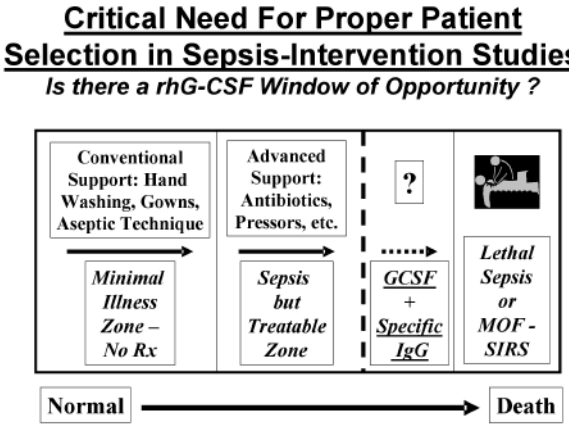

Figure 1. Treatments using combined cellular and humoral therapies for necrotizing enterocolitis or sepsis are critically-dependent on selecting a patient at the correct moment during the progression of their illness.

Neutrophil factors. In VLBW septic-neutropenic and even in preeclampsia-associated neonatal neutropenia, rhG-CSF causes a significant increase in neutrophil cell number.[21,23,24,26,29,40] However, although the quantity of neutrophils is elevated, the function of those cells remains sub-optimal as both phagocytic activity and oxidative burst remain reduced, as does the percent neutrophil population expressing Fcγ and complement receptors in the most severe cases.[21,22,27,28] Under these conditions, the bedside clinician observes a clear improvement in neutrophil cell count on laboratory testing but the patient may be unlikely to accrue a comparatively large increase in phagocytic defense without receptor-competent cells; a theoretical concern that is yet to be tested clinically.

Safety of rhG-CSF cytokine therapy

Effects on neonatal morbidities. The safety profile of rhG-CSF has been fairly well documented in neonates *in vivo*.[22,34,46,47] In the largest randomized U.S. trial of rhG-CSF used to treat symptomatic, septic neonates, there was no significant difference in side effects in patients receiving placebo or study drug[46] or in other series.[47] Moreover, in a newborn piglet model of bronchopulmonary dysplasia, although there was a robust increase in the peripheral blood absolute neutrophil

Table 1. Effects of rhG-CSF on Cytokine, Growth Factor and Hormone Responses in Human Studies.

Time of Administration	Pro-inflammatory	No Change	Anti-inflammatory
rhG-CSF Treatment Given Before Insult [e.g. 1st dose ≥ 8 hours prior]	[32] ↑ Granulocyte and Monocyte counts ↑ IL-6 ↑ IL-8 ↑ GCSF	-IL-1β -IL-2 -TGFβ1	↑ IL-10 ↑↑ IL-1ra ↑↑ sTNF- receptors p55 and p75 ↓ GMCSF ↓ Interferon- γ ↓TNF-α (except LPS stim)
	[41] ↑ Granulocyte and Monocyte counts [transient ↓ after endotoxin administration] ↑ TNF-α	-Cortisol Level -IL-1β -IL-6	↑ IL-1ra ↑ sTNF- receptors p55 and p75
	[39] ↑ Granulocyte and Monocyte counts	-GCSF -IL-1ra -IL-6 -TNF- α	↑ IL-1ra ↑ sTNF- receptors p55 and p75 ↓ IL-8
	[38] ↑ IFN-γ ↑ IL-2 ↑ IL-6 ↑ Neutrophil count ↑ TNF-α		↑↑ IL-1 ra ↑↑ sTNF- receptors p55 and p75 ↓ CRP Levels ↓ GCSF Levels
	[37] ↑ GCSF Level ↑ Neutrophil count	-IL-1ra -IL-6 -Oxidative Burst -Phagocytic Activity -sTNF- receptors p55 and p75 -TNF-α	
rhG-CSF Treatment Either Concurrently or After Insult	[39] ↑ TNF-α ↑ GCSF ↑ IL-6 ↑ IL-8 ↑ Granulocyte and Monocyte counts		↑ IL-1ra ↑ sTNF- receptors p55 and p75
	[40] ↑ ANC ↑ Leukocytosis	-IL-1β -TNF-α	↓ CRP ↓ IL-6 ↓ IL-8
	[25] ↑ANC ↑ NK Cells, Monocytes and Lymphocytes		↓ IL-12 ↓ Interferon- γ ↓TNF-α
	[30] ↑ ANC	-GCSF Level	↓ TNF-α

count (ANC) upon exposure to intravenous rhG-CSF, there was no significant increase in the ANC in bronchial-alveolar lavage fluid or in other markers of lung injury; consistent with most other clinical reports using rhG-CSF[22,46,47] except one small series where BPD may be elevated.[35] Furthermore, rhG-CSF has anti-inflammatory properties as evident by its lowering of neonatal TNFα blood levels.[28,30,31] Thus, there is virtually no evidence to date that an rhG-CSF-induced proinflammatory state may actually be harmful to the human neonate for increasing chronic lung disease, NEC, periventricular leukomalacia, retinopathy of prematurity or worsening of any other clinical condition.[46,47] Experience using rhG-CSF over several years to treat cyclic neutropenia or in neutropenic cancer patients would support this contention of no harmful effects.[34,47] In fact, there is evidence that there may even be fewer complications of prematurity in the ensuing weeks after receiving rhG-CSF for sepsis (see NEC impact below).[46,49]

Effects on mortality. In spite of these compelling clinical and laboratory observations, when reviewed as a meta-analysis, rhG-CSF has not been shown to offer a significant increase in survival in any neonatal clinical condition to date.[47] On the other hand, in a sub-group analysis of these same reports, there is a trend toward lower mortality in both neutropenic patients and in those neonates with birth weight < 2

Table 2. rhG-CSF therapeutic intervention trials and NEC.

Study and year	*Study Type*	*Route Of Admin*	*N*	*Mean Birth Weight (gr.)*	*NEC Dx At Entry*	*Death from NEC*	*N*	*Mean Birth Weight (gr.)*	*NEC Dx at entry*	*Death from NEC*
Kocherlakota & LaGamma 1997[24]	HC	IV	14	1744	7	2	11	1547	6	2
La Gamma et al. 2000[46]	RPC	IV	65	865	6	0	71	835	9	7
Ahmad A et al. 2002[35]	RPC	IV	10 (G)	1050	5	1	8	957	1	0
Total no. of patients	--	--	89	--	18	3 (17%)*	90	--	16	9 (56%)

*HC = historical controls; RPC = randomized placebo controlled; IV= intravenous: *p < 0.04 by Chi square analysis.*

kilograms; as well as in patients with NEC who were treated with rhG-CSF at the time of their diagnosis (Table 2). In this NEC subset of data extracted from the reports indicated, the reduction of NEC-related mortality was notable in its magnitude as well as being seen across several studies achieving statistical significance. A randomized trial examining intravenous rhG-CSF treatment of NEC as the entry criteria has never been done; however, oral use of the cytokine is encouraging and showed benefit for a small group of randomized subjects in slowing progression beyond Bell's stage I.[50]

NEC: the "ideal" clinical scenario for cytokine therapy

The *ideal* clinical scenario for the use of rhG-CSF would occur in i) a disease process in which there is an associated serious morbidity and mortality, ii) where there are clearly defined objective clinical criteria for the early diagnosis of a graded severity of illness, iii) that an associated infectious and inflammatory component exists, and most significantly, iv) that there is enough time after diagnosis of the condition to allow the study medication to work (Figure 1). Furthermore, v) the disease process itself must be frequent enough to enroll a sufficient number of study subjects in a reasonable amount of time to maintain clinical equipoise to test whether there is an effect.

Each of thee issues has hampered our understanding and ability to identify the best candidate (other than neutropenic patients) for augmented immunotherapy using rhG-CSF.[47] However, in carefully reviewing various reports, rhG-CSF has a protective and immune system boosting effect as well as significant anti-inflammatory effects in both human and animal models (Table 1).[36,45,51] Moreover, this beneficial effect was most pronounced in models of monomicrobial or polymicrobial peritonitis, where administration of rhG-CSF either before or after the infectious challenge resulted in better outcomes.[36-38] Thus, a clearly important issue of experimental design centers not only on the proper identification of the ideal candidate but also the precise timing of when intervention is initiated (Figure 1).

When rhG-CSF effects are viewed in conjunction with immature mucosal barrier immune defenses, NEC is clearly an ideal candidate disease process for further study (Figure 2). NEC characteristically involves i) serious infection, ii) is often associated with neutropenia and inflammation that causes iii) destruction of the bowel due to high circulating and enterohepatic levels of TNFα along with other inflammatory cytokines like IL6.[9,14,16,17] The release of markers/effectors like IL6 is especially dramatic when infection progresses from the abdominal region to systemic illness.[10] The clinical course of NEC is predictable in its progression over a series of days and shows pathogneumonic findings over that time sequence (Bell's staging).[52,54] Moreover, NEC exhibits objective evidence discernable on abdominal radiographs as pneumatosis intestinalis.

When NEC is complicated by pneumatosis intestinalis, it increases the chances that the neonate will suffer an intestinal perforation and die from overwhelming sepsis.[1,52-55] On the other hand, pneumatosis typically occurs 12-48 hours after NEC's presenting signs and 1-4 days prior to perforation.[53-55] Therefore, the presence of pneumatosis intestinalis would apparently allow a clear and objective clinical entry point for the initiation of rhG-CSF therapy. The patient is already critically ill with a serious infectious problem, yet is highly likely to live long enough under conventional support to allow the rhG-CSF intervention to have a logical chance to work (Figures 1 and 2). This cytokine drug has never been directly studied in this way.

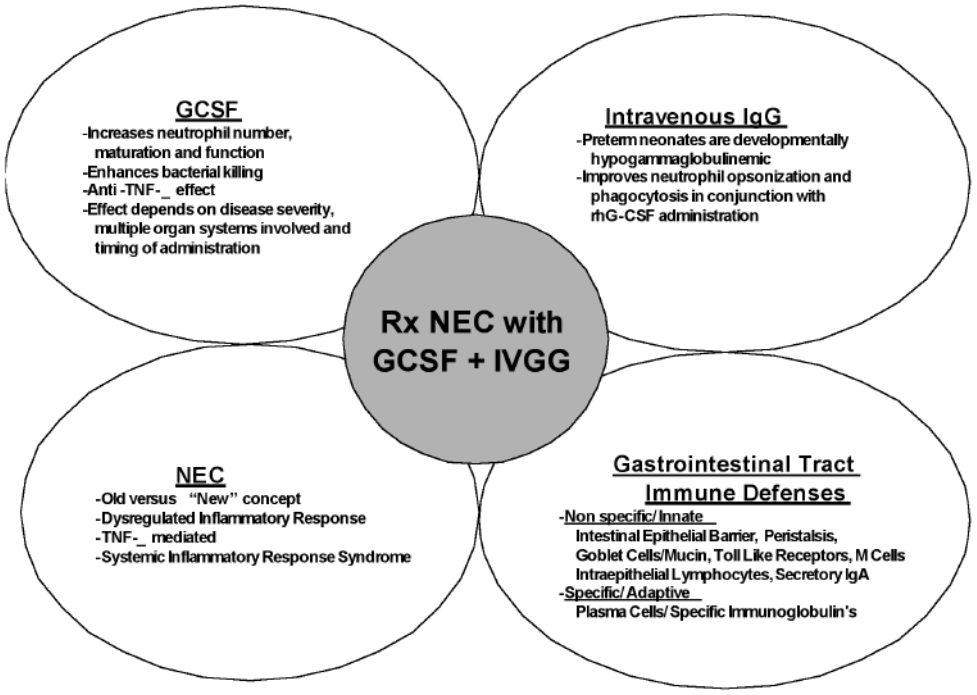

Figure 2. Overlapping arguments in support of combined cellular and humoral therapy for necrotizing enterocolitis.

What do we know about G-CSF and NEC in clinical trials?

Currently, there have been no clinical trials that have directly addressed the use of intravenous use of rhG-CSF in neonates with NEC and one oral therapy for early stage I.[47,50] As described earlier, there appears to be a benefit of giving rhG-CSF to a certain subset of neonates with neutropenia and in the context of the current review, NEC. In the largest interventional trial of rhG-CSF and late onset sepsis in neonates (n = 71 placebo and n = 65 rhG-CSF treated symptomatic subjects, Amgen Protocol 950212), there was no significant difference in overall mortality (14%, 10/71 placebo vs. 14% 9/65 rhG-CSF group) or in the incidence of NEC prior to initiating therapy (13%. 9/71 placebo vs. 9% 6/65 rhG-CSF group).[46] However, in a sub-group analysis of only NEC subjects extracted from this and two other clinical trials (where intravenous rhG-CSF was given at the time of diagnosis of NEC), there was a statistically significant decrease in mortality in the pooled data (Table 2: 56%, 9/16 placebo/vehicle vs. 17%, 3/18 rhG-CSF group; $p < 0.04$). Recently, the use of enteral rhG-CSF in stage I NEC showed encouraging evidence that proper timing of therapy can benefit the neonate by limiting progression to more severe stages (0%, 0/8 rhG-CSF vs 50%, 5/10 vehicle, $p < 0.05$), unfortunately the study was limited due to sample size.[50]

It is also interesting to note that in 2 studies where rhG-CSF was given several weeks earlier in the postnatal period for sepsis-interventional purposes, there were fewer subsequent late onset nosocomial infections in both (e.g. Amgen Protocol 950212: 10% in rhG-CSF vs. 24% in placebo subjects and in Miura[49]: 9%, 2/22 vs. 41%, 9/22 $p < 0.02$) yet no affect on overall mortality.[46,49] While these trials were not specifically designed or powered to test the effect of rhG-CSF on NEC or on future infectious complications, in the context of the current review, collectively, these reports on NEC and late infections illuminate an intriguing possibility of benefit that requires future investigation to confirm.

What about combining rhG-CSF and intravenous IgG (IVIG)?

VLBW neonates born prematurely (i.e. prior to 30 weeks gestational age) have compromised humoral defenses due to a significant hypogammaglobulinemia occurring from being born prior to the facilitated transfer of IgG class antibodies across the placenta from the mother; a problem that can exist for months after birth.[22,56] Previous research suggests that there is an improvement in both phagocytosis and oxidative burst following administration of exogenous gamma globulin *in vitro*.[57,58] In fact, when given as prophylaxis to ELBW preterm infants, IVIG was shown to decrease the rate of sepsis and serious infection.[58,59] Furthermore, when given to neonates in septic shock, there was a trend towards a reduction in mortality although not statistically significant.[60] What is significant about these findings is that neutrophil killing capacity can be augmented by prior *in vivo* treatment

of neonates with rhG-CSF in the presence of pooled antibody and that this corresponds to evidence of improved clinical outcomes.[21,22,47,58-60]

Studies in VLBW neonates have demonstrated that treatment with rhG-CSF (10 μg/kg/d intravenously in D5W infused over 30 minutes; at a final concentration of 15 μg/ml to prevent protein denaturation - as recommended by Amgen Corp, Thousand Oaks, CA) for as little as 3 days, improves neutrophil cell number, increases the population of neutrophil's expressing Fcγ and complement receptors and enhances opsonization of bacteria.[21,22,26-29] Whether these effects of combined intravenous rhG-CSF + IVGG therapy would prove beneficial for patients with the early stages of NEC has never been tested except as part of other trials (Table 2) or as enteral use in stage I NEC.[50]

When is the optimal time to initiate rhG-CSF therapy?

Historical approach to timing rhG-CSF for sepsis

Improving outcome in critically ill patients requires that the subject be sufficiently ill to have a clearly discernable benefit, typically mortality. In an adult trial of community acquired pneumonia, respiratory symptoms abated earlier in non-neutropenic subjects yet no mortality benefit was accrued.[61] In our opinion, similar to adult non-neutropenia studies, in each of the foregoing neonatal trials using rhG-CSF (Table 2) and reviewed in reference number 47, the criteria for intervention was likely either too late to be helpful for a rapidly progressing deterioration or the subjects were not sufficiently ill to accrue a mortality benefit (e.g. treated subjects were outcome failure due to low mortality associated with S. epi sepsis). This suggests there are certain conditions like peritonitis,[36-38,62] septicemia,[22,45] or NEC (Table 2)[50] that are likely to accrue benefit in non-neutropenic subjects if the proper timing and combination of clinical features for intervention coincide (Figure 1 and 2).

Timing of rhG-CSF for treatment of NEC

Therefore, by building on the observations of the previous clinical sepsis trials and in view of the foregoing immune-defense issues, as well as the time necessary for rhG-CSF effects to occur and ultimately, in view of the natural progression and deterioration during the clinical course of NEC, in our opinion, the optimal moment for initiating treatment would be at the stage of unambiguous diagnosis of a moderately severe form of NEC, prior to progression to intestinal perforation with peritonitis and before overwhelming septic shock (Figure 1). The pragmatic reasons behind this interpretation are important to consider.

Since the clinical triad characteristic of NEC consists of i) abdominal distention, ii) bilious gastric residuals, and iii) bloody stools are lacking in easily quantifiable graded endpoints, pneumatosis intestinalis seen on abdominal radiograph stands-out as a more objective measure of severity of illness (that typically appears 1-2 days before further progression; Bell's stage IIb). Moreover, when present, pneumatosis signifies an increased chance that the neonate will suffer an intestinal perforation and die from overwhelming peritonitis and systemic sepsis.[52-55] Thus, in our opinion based on animal and human data plus other pragmatic considerations in the timing of NICU management strategies (e.g. abdominal radiographs every 6-8 hours, etc.); beginning treatment with rhG-CSF + IVGG upon diagnosis of pneumatosis intestinalis represents an ideal synthesis of biology and medicine with practical reality. This or a related approach warrants an interventional trial to test its veracity.

Closing comments on rhG-CSF cytokine research

NEC is a multi-factorial disease that, when treated as an infectious process, still has a significantly poor lifelong gut and neurodevelopmental morbidity plus a high associated neonatal mortality. When viewed from the perspective of the *new* NEC, the pathological progression leading to tissue destruction results from a dysregulated, but potent inflammatory reaction to invading bacteria.

As ELBW neonates continue to survive at earlier gestational ages at ever increasing rates, we have a moral obligation to explore and develop interventions that can decrease neonatal morbidity and mortality from relatively common life threatening diseases like infections and NEC. Moreover, improved management of infections is likely to contribute to enhanced neurodevelopmental outcome of these very small and fragile human newborns. rhG-CSF + IVGG may just be one of those interventions that warrant a well designed therapeutic trial to test this point.

We speculate that the ability to increase bacterial killing capacity while minimizing the tissue destructive capacity of the proinflammatory response should result in improved survival and less short gut injury from NEC.

What is needed to move this speculation forward using current knowledge is a randomized, blinded placebo controlled trial in VLBW neonates with early stages (i.e. <Bell's Stage IIb) of NEC, who are treated with either placebo or a combination of rhG-CSF + IVGG to supplement conventional standard-of-care support like antibiotics, fluid management and cessation of enteral fluids. In our opinion, the greatest potential shortcoming in this area is not the potential for no beneficial effects of combined therapy but in never having the opportunity to evaluate the approach properly.

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