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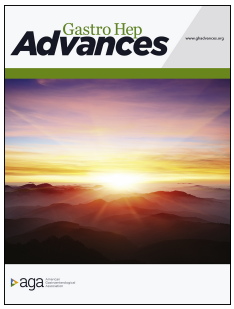


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# Non-invasive faecal cytokine and microbiome profiles predict commencement of necrotizing enterocolitis in a proof-of-concept study

**Short title:** NEC prediction by faecal cytokines

Christian Zenner[1], Lisa Chalklen[2], Helena Adjei[2,] Matthew J. Dalby[2,] Suparna Mitra[2,3], Emma Cornwell[4], Alex Shaw[4], Kathleen Sim[4], J. Simon Kroll[4], Lindsay J. Hall[1,2,5]

**Affiliations:** [1]Technical University of Munich, School of Sciences, Intestinal Microbiome. [2]Quadram Institute Biosciences, Gut Microbes & Health; [3]Leeds Institute of Medical Research, University of Leeds; [4]Imperial College London, Department of Medicine; Section of Paediatric Infectious Disease; [5]University of East Anglia; Norwich Medical School

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**Abbreviations:** DOL: day of life, NEC: Necrotizing enterocolitis, NICU: neonatal intensive care unit, OTU: operational taxonomic unit, VLBW: very low birthweight

**Correspondence:** Prof. Lindsay Hall, Technical University of Munich, School of Sciences, Intestinal Microbiome, Weihenstephaner Berg 3, 85354 Freising, Lindsay.hall@tum.de, +49 (0) 8161-71-3516

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**Author contributions:** Dr Zenner drafted the initial manuscript, interpreted the data, carried out final analyses, and critically reviewed and revised the manuscript. Lisa Chalken and Helena Adjei collected data and carried out initial analyses. Dr Dalby carried out initial analyses, interpreted the data, and critically reviewed and revised the manuscript. Dr Mitra and Emma Cornwell carried out initial analyses. Dr Sim conceptualized and designed the study, coordinated and supervised data collection, collected data, carried out initial analyses, and critically reviewed and revised the manuscript. Dr Shaw and Prof Kroll conceptualized and designed the study, coordinated and supervised data collection, and critically reviewed and revised the manuscript for important intellectual content. Prof Hall drafted the initial manuscript, conceptualized and designed the study, coordinated and supervised data collection, critically reviewed and revised the manuscript, and supervised the project. All authors

approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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## Abstract

**Background & Aims:** Necrotizing enterocolitis (NEC) is a life-threatening disease, and the most common gastrointestinal emergency in premature infants. Accurate early diagnosis is challenging. Modified Bell's staging is routinely used to guide diagnosis, but early diagnostic signs are non-specific, potentially leading to unobserved disease progression, which is problematic given the often rapid deterioration observed. We investigated faecal cytokine levels, coupled with gut microbiota profiles, as a non-invasive method to discover specific NEC-associated signatures that can be applied as potential diagnostic markers.

**Methods:** Premature babies born below 32 weeks of gestation were admitted to the 2-site neonatal intensive care unit (NICU) of Imperial College hospitals (St. Mary's or Queen Charlotte's & Chelsea) between January 2011 and December 2012. During the NICU stay, expert neonatologist grouped individuals by modified Bell's staging (healthy, NEC1, NEC2/3) and faecal samples from diapers were collected consecutively. Microbiota profiles were assessed by 16S rRNA gene amplicon sequencing and cytokine concentrations were measured by V-Plex multiplex assays.

**Results:** Early evaluation of microbiota profiles revealed only minor differences. However, at later time points, significant changes in microbiota structure were observed for Bacillota (adj. p=0.0396), with *Enterococcus* being the least abundant in Bell stage 2/3 NEC. Evaluation of faecal cytokine levels revealed significantly higher concentrations of IL-1α (p=0.045), IL-5 (p=0.0074), and IL-10 (p=0.032) in Bell stage 1 NEC compared to healthy individuals.

**Conclusions:** Differences in certain faecal cytokine profiles in patients with NEC indicate their potential use as diagnostic biomarkers to facilitate earlier diagnosis. Additionally, associations between microbial and cytokine profiles contribute to improving knowledge about NEC pathogenesis.

**Keywords:** preterm infants; non-invasive biomarkers; cytokines

## Introduction:

Necrotizing enterocolitis (NEC) is a life-threatening disease that primarily affects very low birthweight (VLBW) preterm infants born weighing less than 1500g.[1] The estimated average incidence of NEC cases across 27 studies conducted worldwide is ~7% among VLBW infants.[2] However, contrasting regional differences are reported in the literature, with a prevalence of NEC of 25.4% for enteral fed and low birthweight infants admitted to public hospitals in Addis Ababa, Ethiopia,[3] compared to only 1.6% in VLBW infants in Japan.[4]

Although clinical manifestations of the disease have been known since the 1940s,[5] its aetiology remains incompletely understood and is often described as multifactorial.[6] The most important contributing factors for the development of NEC is prematurity, including low birthweight and low gestational age.[7,8] Other potential factors are formula feeding,[9] prolonged parenteral feeding,[10] and an abnormal microbial colonization,[11] potentially leading to a perturbed state in the premature intestine.[12,13] The gut of vaginally delivered and breast-fed term babies is typically dominated by bacteria of the genus *Bifidobacterium,*[14,15] whereas preterm infants, who are often born by caesarean section and receive antibiotic treatment, are populated by genera such as *Enterococcus, Klebsiella,* and *Enterobacter.*[15] Overgrowth of these potentially pathogenic bacteria within the gut microbiota, and/or colonisation of the preterm gut by hospital-acquired pathogens plays a crucial role in the onset of NEC.[16] Frequently detected bacteria occurring in association with NEC include *Clostridium spp., Enterococcus spp., Escherichia coli, Pseudomonas aeruginosa, Salmonella spp., Klebsiella spp., and Staphylococcus spp..* [16] These potential pathogens can be partially suppressed by supplementation with probiotics including *Bifidobacterium* spp. and

*Lactobacillus spp*., which is also associated with a 50% reduction in NEC incidence.[17,18]

The prognosis for infants diagnosed with NEC is poor, with survivors at risk of long-term neurodevelopmental limitations and growth restrictions.[19-21] The Bell staging criteria were introduced in 1978 to classify different stages of illness severity, suggest disease management, and guide treatment,[22] and were later refined in 1986.[23] Various other staging criteria for NEC have been proposed by expert neonatologists, including the Vermont Oxford Network definition, Centers for Disease Control and Prevention definition, Gestational Age-Specific Case Definition of NEC, Two of 3 rule, Stanford NEC score, and International Neonatal Consortium NEC workgroup definition. However, modified Bell staging remains the most frequently used,[24] despite questions remaining about its reliability.[25]

Researchers have focused on additional measures including the infant gut microbiome that could better predict cases of NEC. Dobbler et al. reported that both lower microbial diversity and bacteria belonging to the family *Enterobacteriaceae* correlated with NEC, with *Citrobacter koseri* and *Klebsiella pneumoniae* being the most abundant species within this family.[26] Low bacterial diversity in combination with high abundance of Pseudomonadota prior to the onset or at diagnosis of NEC has been confirmed by other studies.[27-33] In contrast, Cassir et al. showed a strong association between *Clostridium butyricum* and NEC incidence and identified cytotoxic activity in the supernatant of cultured C. *butyricum* isolates.[34] The role of the gut microbiota in the development of NEC remains complex and is likely to be dependent on NICU location (i.e. circulating nosocomial pathogens) and underlying individual microbial communities present in the preterm infant gut.

Human milk oligosaccharides (HMOs) are now a topic of research interest due to their role in feeding specific bacteria, especially *Bifidobacterium*, which are not typically abundant in the preterm infant gut microbiota.[35] Sodhi et al. recently suggested the HMOs 2'-fucosyllactose and 6'-sialylactose protect against the development of NEC through the inhibition of Toll-like receptor (TLR) 4 signalling.[36] Masi et al. showed that the concentration of the HMO disialyllacto-N-tetraose (DSLNT) was lower in the breast milk of mothers of NEC infants and associated with a lower abundance of *Bifidobacterium* species.[37]

The role of cytokines and pro-inflammatory mediators in NEC have been extensively reviewed. In particular, increased levels of TLR 4, IL-18, IFNγ, Platelet-activating factor (PAF), IL-6, IL-8, IL-1β, and NF-κB have been linked to NEC severity, while deficiencies of TLR 9, IL-1R8, IL1-Ra, $TGF\beta_2$, PAF-acetylhydrolase, and IL-10 pave the way for NEC-associated inflammation.[38]

Novel approaches are needed to provide guidance to clinicians and healthcare professionals to select the appropriate therapy.[39] Previous studies have aimed to find suitable and robust biomarkers that may be used to predict NEC, including platelet counts,[40] levels of C-reactive protein,[41] serum amyloid A,[42] claudin proteins,[43] plasma citrulline,[44,45] endogenous RNA molecules,[46] volatile organic compounds,[47] lipocalin-2 and calprotectin.[48] Systemic cytokine concentrations have been suggested as potential biomarkers for the prediction of NEC and disease outcome.[38,49-52] Rising cytokine levels were highly specific for the diagnosis of neonatal sepsis, but additional (non-invasively assayed) biomarkers are needed for high specificity and sensitivity to predict NEC.[53]

In this study we evaluate the gut microbiota profiles and the measurement of faecal cytokine levels as a rapid and non-invasive tool for the early detection of NEC.

## Methods:

### Study design

Samples were provided from a study published in 2015.[13] This exploratory study included infants born <32 weeks of gestation, without severe congenital birth defects. Infants were admitted to the Imperial College Healthcare NHS Trust neonatal intensive care unit (NICU) between January 2011 and December 2012. In total, 39 individuals were included in the study (Bell stage 1: n=7; Bell stage 2/3: n=11; healthy controls: n=21). Probiotics and H2-receptor antagonists were not used within the NICU at the time of recruitment and sampling. Patient IDs were blinded. Only members of this research group had access to patient information.

### Sample collection

Research nurses collected faecal samples from diapers using a sterile spatula, placed in sterile DNase-, RNase- free Eppendorf tubes, stored in a -20°C freezer on the neonatal unit within 2 hours of collection, and stored at -80°C within 5 days. NEC cases were diagnosed by the attending neonatal consultant and confirmed by an independent neonatologist (Bell stage 2/3 by Bells' modified staging criteria). Multiple samples were taken from individuals included in the study during their stay in NICU. Sample numbers were as follows: Bell stage 1 NEC n=23; Bell stage 2/3 NEC n=47; healthy controls n=86.

### Cytokine measurement

One gram of faecal material was homogenized with one ml PBS using a FastPrep® Bead Beater (4.0m/s, 3min), centrifuged (14,000rpm, 15min) and 25µl of supernatant was used for the assay. Samples were analysed using MULTI-SPOT™ plates, MESO Quickplex SQ120 and discovery workbench software according to the manufacturer's

protocol. Pre-coated immunoassays V-PLEX Proinflammatory Panel 1 (human) and V-PLEX Cytokine Panel 1 (human) were used to detect a set of 20 different cytokines: IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNFα, GMCSF, IL-1α, IL-5, IL-7, IL-12p40, IL-15, IL-16, IL-17A, TNFβ, and VEGF-A. If cytokine values drastically exceeded comparable sample values, the sample was excluded from the analysis. Samples not reaching the lower limit of detection were generally considered as very low and were taken into account without statistical resolving.

**DNA extraction, 16S rRNA gene amplification and sequencing**

Information about sample preparation, gene amplification and sequencing are documented elsewhere.[13]

**16S rRNA sequencing data analysis**

Roche 454 pyrosequencing data in standard flowgram format was transcribed to fastq format using Bio.SeqIO.SffIO module in biopython. Single fastq files were remultiplexed using the perl script remultiplexor (available at https://www.imngs.org). Remultiplexed sequencing data was processed with the integrated microbial NGS platform (IMNGS),[73] with parameters set as follows: Barcode mismatches, 1; quality trim score, 10; min. read length 100bp; max. read length 1000bp; max. rate of expected error, 2% of sequence length; min. alignment id 70%. Operational taxonomic units (OTUs) were clustered at 97% sequence similarity, using a cutoff of ≥0.25% relative abundance in at least one sample. Data was further analysed and visualized using RHEA,[74] a modular pipeline for microbial profiling, using R(v4.0.5) and Rstudio (v1.4.1106). Samples not achieving specific QC criteria (>1000 reads/sample; rarefaction curves Suppl. Fig 1) were excluded from the analysis, leading to reduced sample numbers: Bell 1 NEC 1 n=18; Bell 2 NEC 2/3 n=41; healthy controls n=63.

122 **Statistical testing**

123 Cytokine profiles were evaluated pairwise between groups using Mann-Whitney-U
124 Test. The following methods were applied for 16S rRNA gene amplicon data: Fishers
125 Exact Test, Wilcoxon Rank Sum, and Kruskal-Wallis Rank Sum Test. The method used
126 is referenced in the respective paragraph or figure. Multidimensional scaling plots are
127 based on generalized UniFrac distances. The p-values were calculated using
128 PERMANOVA.

129 All authors had access to the study data and had reviewed and approved the final
130 manuscript.

131

132 **Results:**

133 A total of 39 preterm infants with a gestational age <32 weeks were included in this
134 study, 7 were diagnosed with Bell stage 1 NEC, 11 were diagnosed with Bell stage 2/3
135 NEC and 21 were healthy controls (not diagnosed with NEC). Detailed information
136 about participants and sample numbers are represented in table 1. All but two babies
137 received a first course of antibiotics from birth onwards. Faecal samples from diapers
138 were collected longitudinally during their NICU stay.

***Table 1**: Cohort information of study participants*

| | **All** | **NEC2/3** | **NEC1** | **healthy** |
|---|---|---|---|---|
| **Number of individuals** | 39 | 11 | 7 | 21 |
| **Received antibiotics** | 37 | 11 | 7 | 19 |

| | | | | |
|---|---|---|---|---|
| **Received additional formula feeding** | 6 | 1 | 3 | 2 |
| **Received mechanical ventilation** | 25 | 10 | 4 | 11 |
| **DOL at NEC diagnosis (mean, min-max)** | - | 29 (9-43) | 29 (17-82) | - |
| **Samples used for microbiota analysis >1000 reads** | 122 | 41 | 18 | 63 |
| **Samples used for cytokine analysis** | 156 | 47 | 23 | 86 |
| **Gestational age (mean ± StDev)** | 27+1 (190d) ± 2+1 (15d) | 26+6 (188d) ± 2+1 (15d) | 27+2 (191d) ± 0+5 (5d) | 27+2 (191d) ± 2+4 (18d) |
| **Birthweight (mean ± StDev)** | 922g ± 283g | 843g ± 204g | 937g ± 140g | 959g ± 348g |
| **Gender** | f=15<br>m=24 | f=4<br>m=7 | f=2<br>m=5 | f=9<br>m=12 |

Characterization of the neonatal gut microbiome of these preterm infants was carried out using 16S rRNA gene amplicon sequencing. An average of 7.8 (±3.6) OTUs (a proxy for bacterial species) was detected across the three infant groups. Healthy infants contained a mean of 8.4 OTUs/sample, which was lower at 7.6 OTUs/sample in the NEC1 infants and 6.9 OTUs/sample in the NEC2/3 infants, but the differences were not statistically significant (Fig. 1A). The multi-dimensional-scaling (MDS) plot of

microbial profiles representing *beta*-diversity showed no significant differences across the three study groups (p=0.106) (Fig. 1B). To detect age-dependant differences, samples were split up into four different time points (TP1: 0-10 days of life (DOL), TP2: 11-20 DOL, TP3: 21-30 DOL, TP4: 31-Maximum age). Significant differences in the *beta*-diversity were detected at time point 4 in the MDS plot (p=0.02) (Fig. 1B). By comparing the groups at taxonomic levels, the only detected significant differences were between Bell stage 2/3 and healthy controls for the order Bifidobacteriales, including family Bifidobacteriaceae and genus *Bifidobacterium* (adj. p=0.0204 for all three taxonomic levels, Fisher's exact Test, pairwise comparison) (Fig. 1C). At all taxonomic levels, no significant differences were detected at TP1 and TP2. At TP3, a significantly higher relative abundance of *Escherichia-Shigella* in Bell 2/3 was detected compared to the healthy group (p=0.0003, Wilcoxon Rank Sum Test, pairwise, data not shown). At TP4, the microbiota profiles became more clearly different. The phylum Bacillota was lower in Bell 2/3 (mean rel. abundance 10.0 %) compared to Bell 1 (mean rel. abundance 18.1%) and healthy (mean rel. abundance 15.6%) (adj. p=0.0396, Wilcoxon Rank Sum Test, pairwise comparison, equal p-value for both comparisons) (Fig. 1C). Differences in Bacillota were mostly represented by differences in the family Enterococcaceae and the subordinate genus *Enterococcus* (NEC1 vs. NEC2/3 adj. p=0.0142; NEC2/3 vs. healthy adj. p=0.0096, Wilcoxon Rank Sum Test, pairwise, values are equal for family and genus) (Fig. 1C). Individuals that developed NEC were further compared with age-matched healthy preterm babies, with phylum profiles measured longitudinally until NEC diagnosis. Only two NEC babies displayed high Actinomycetota abundance (N15, N18), whilst this phylum was better represented in the healthy control babies. Bacteroidota was generally underrepresented in the studied individuals. Fusobacteria were also rare, and only found in one control baby at one time point (C27_3) (Fig. 1D).

We also explored factors that could potentially impact microbiota profiles, e.g. condition at birth (APGAR), total parenteral nutrition (TPN), need for mechanical ventilation, feeding type, and antibiotics usage. APGAR score can be used as prognostic indicator for neonatal death in preterm infants,[54] however differences between study groups were minor and not significant. TPN was performed for all but four babies, and has been previously shown to impact the gut microbiota.[55] In this study, all samples were taken after TPN (average length of TPN NEC1 5.3 days, NEC2/3 5.7 days, healthy 6.1 days) was finished, thus we were not able to determine if there were any TPN-associated microbial changes. The need for mechanical ventilation was heterogeneous across all groups. We did not observe any significant differences in NEC1 and NEC2/3 groups. However, within the healthy group, and only analyzing samples between 9-21 DOL to reduce the age bias, microbial richness was significantly elevated in the non-ventilated group ($p = 0.0087$). In terms of feeding type, only six individuals (NEC2/3 n=1; NEC1 n=3; healthy n=2) received formula milk ('top-up') in addition to maternal and/or donor breast milk, and we did not observed any clear differences. We did observe some changes in one individual in the Bell stage 1 group, from 12 DOL to 14 DOL during formula feeding (rise of Actinomycetota by 5%, an increase of Pseudomonadota by 13% and a decrease of Bacillota by 18%), however this is only one individual and these changes may be associated with normal microbiota changes over time. Regarding antibiotics usage, only two individuals (both in the healthy group) did not receive antibiotics during their NICU stay, which correlated with high abundance of Actinomycetota (genus *Bifidobacterium,* at TP2 and TP3).

Faecal cytokine concentrations were then analysed to determine differences in these host-associated immune factors. Pro- and anti-inflammatory cytokines play an important role in the development and progression of NEC and systemic levels are often measured. As NEC is essentially an intestinal disease, cytokine concentrations measured in faeces could be more representative of immune activation in NEC.

In these infants almost all measured cytokine concentrations were significantly higher in the NEC 2/3 group (Fig. 2A). Significant differences between NEC1 and NEC2/3 as well as between NEC2/3 and healthy were observed for IL-2, IL-6, IL-10, IL-12p70, IL-12_IL23p40, IL-13, IL-17A, and Interferon γ ($p \leq 0.0001$, Mann-Whitney-U Test) (Fig. 2A). Significantly higher concentrations in NEC1 compared to healthy were observed for IL-1α ($p=0.045$), IL-10 ($p=0.032$), and IL-5 ($p=0.0074$), suggesting that these could be potential markers for the onset and development of NEC. The concentration of these cytokines was further investigated at each time point (Fig. 2B). For IL-1α, significant differences were detected at TP1 (NEC1 vs. NEC2/3, $p=0.0307$, and healthy vs. NEC2/3, $p=0.0177$) and TP4 (healthy vs NEC1, $p=0.0057$, and healthy vs. NEC2/3, $p=0.001$). For IL-5, significant differences were observed at TP1 (NEC1 vs. NEC2/3, $p=0.0106$, and healthy vs. NEC2/3, $p=0.0004$), TP2 (healthy vs. NEC1, $p=0.0115$, and healthy vs. NEC2/3, $p=0.004$), TP3 (healthy vs. NEC2/3, $p=0.0228$), and TP4 (healthy vs. NEC2/3, $p=0.0432$). Significantly higher levels of IL-10 were found in the NEC2/3 group at all time points, TP1 (NEC1 vs. NEC2/3, $p=0.0045$, healthy vs. NEC1, $p=0.031$, healthy vs. NEC2/3, $p<0.0001$), TP2 (NEC1 vs. NEC2/3, $p<0.0001$, healthy vs. NEC2/3, $p<0.0001$), TP3 (healthy vs. NEC2/3, $p<0.0001$), and TP4 (NEC1 vs. NEC2/3, $p=0.0275$, healthy vs. NEC2/3, $p=0.0003$).

Cytokine profiles were further analysed 5-10 days before the date that NEC was diagnosed and compared with age matched healthy preterm infants (+/- 1 day

difference). Significantly higher levels of IL-10 (p=0.0013), IL-13 (p=0.0062), IL-4 (p=0.0293), and IL-6 (p=0.0322) were measured in the Bell stage 2/3 group compared to healthy controls (Fig. 2C). The same analysis was performed 11-17 days before NEC diagnosis with significant differences again detected for IL-10 (p=0.0004), IL-13 (p=0.0335) and IL-6 (p=0.0122), with additional cytokines IL-12p70 (p=0.0294), IL-17A (p=0.0004), IL-5 (p=0.0294), and TNFβ (p=0.0066) also differentiating between NEC and healthy controls (Fig.2D).

When we analyzed cytokine profiles with additional clinical variables (as outlined above), we only observed significant differences for mechanical ventilation within the healthy group (samples between 9-21 DOL were analyzed to reduce the age bias) for IL-15, which was significantly higher in the ventilated group (p = 0.0427).

## Discussion:

Although known for decades, NEC remains a major challenge for neonatologists, given the abrupt onset and rapid progression of the disease. Targeted treatments are still lacking, leading to high mortality rates and leaving survivors with severe long-term disabilities. Prompt timing of treatment is crucial to maximize the chance of survival. In this study, we investigated the preterm infant gut microbiome in combination with faecal cytokine levels to shed light on disease progression. The preterm intestinal microbiota differs greatly from that of term infants: the number of species present is reduced, patterns of colonization are disrupted and the abundance of pathogenic bacteria is increased.[56-58] Many studies have reported that reduced gut bacterial diversity is a risk factor for the onset of NEC.[26,28,31,59] In our study, samples from the NEC 2/3 group contained the lowest number of OTUs per sample (mean of 6.9), but compared to the other study groups differences were minor and not significant. In terms of taxonomic differences, an enrichment of Pseudomonadota and a reduction of Bacillota and Bacteroidota has often been associated with NEC development.[12,60] However, this was not observed in our study results, with similarly high levels of Pseudomonadota found in all study groups. We do detect significantly lower levels of Bacillota in NEC 2/3 infants at time point (TP4), representing higher Pseudomonadota levels, but this was only the case for infants older than 31 days and was not associated with NEC. A variety of reasons could account for differences between studies, including sampling technique, DNA extraction protocols, selection of 16S variable regions, sequencing technique, bioinformatics pipelines, and databases used, [61,62] making comparisons between studies difficult. As numerous bacteria are potentially associated with NEC, i.e. *Clostridium* spp., *Enterococcus* spp*., Escherichia coli, Pseudomonas aeruginosa, Salmonella* spp*., Klebsiella* spp*., and Staphylococcus* spp.,[16] a single bacterial

signature is not expected. On the other hand, supplementation of probiotic *Bifidobacterium* and *Lactobacillus* is associated with lower abundance of common pathobionts in the preterm gut,[17] which is associated with significantly reduced rates of NEC and late onset sepsis.[18] Indeed, we also observed heathy preterms had higher relative abundance of *Bifidobacterium*, when compared to NEC 2/3 infants, even though these infants did not receive probiotic supplementation. Exploring additional clinical factors revealed that only mechanical ventilation significantly impacted microbial diversity, but this was only observed in 'healthy' premature infants. Surprisingly, we did not see any major differences in formula feeding or antibiotic usage, which would be expected to significantly alter microbiota profiles. This is most likely linked to the low number of formula fed babies, and the fact all were still receiving breast milk thus masking any potential diet-induced changes,[63] and although we observed higher *Bifidobacterium* (which is highly susceptible to antibiotics) in non-antibiotic treated preterms this was only 2 infants. Given the limited number of patients and samples this restricted our ability to do multiple robust comparisons across key clinical parameters.

Although substantial differences in microbiota profiles were not found in this study between NEC infants and healthy controls, the impact of the microbiome on the immune system including signalling molecules such as cytokines is well known.[64] Therefore, the evaluation of faecal cytokine levels is a key aspect of this study. Interestingly, except for IL-1β, the faecal concentrations of all measured cytokines were significantly higher in the NEC 2/3 group compared to healthy controls. IL-1s (including IL-1α and IL-1β) are pro-inflammatory cytokines, produced by a variety of cell types that also induce inflammatory reactions such as tissue damage and fever.[65] IL-1 receptor binding triggers the activation of pro-inflammatory transcription factors

such as NF-κB and AP-1, which can further induce the production of IL-6, Tumor necrosis factor (TNF) and IL-1 itself.[65] Studies on human IL-1α and IL-1β in NEC setting are rare. One study by Benkoe et al. could not identify differences in systemic IL-1β levels in NEC babies compared to healthy controls,[49] concordant with the results of our study. For IL-1α, we could identify significantly higher levels in NEC2/3 compared to NEC1 and healthy at TP1, and significantly higher levels in NEC2/3 and NEC1 compared to healthy at TP1 (Fig. 2B). Interestingly, this finding did not persist during TP2 and TP3, and was again observed at TP4. However, this may be due to the inconsistent number of samples across all time points, which is a limitation of this proof-of-concept study. Another study by Ng et al. showed increased systemic concentrations of IL-2, IL-4, IL-6, IL-10, IFNγ, and TNFα in neonatal septicemia, also including NEC cases,[66] corresponding with the results presented in this study for faecal cytokines. We could also show that local IL-10 levels were significantly higher in NEC2/3 compared to NEC1 and healthy at all time points (Fig. 2B). Additionally, the age matched comparison of babies 5-10 or 11-17 days before NEC diagnosis revealed significantly higher levels of IL-10 (Fig. 2D), indicating an induced protective role of IL-10 to counteract inflammation in the gut. This is also supported by high levels of IL-10 in breast milk,[67] while low levels of IL-10 in breast milk are correlated with NEC incidence.[68] IL-5 primarily promotes activation, survival and adhesion of eosinophils, and is therefore elevated in allergy and parasitosis.[69] Interestingly, we observed significantly higher IL-5 concentrations in NEC2/3 at all time points (Fig. 2B), suggesting a hyper-inflammatory state with involvement of eosinophils, coinciding with a study from 2000.[70] While IL-4 and IL-5 were involved in NEC progression in rats,[71] Benkoe et al. demonstrated significantly lower IL-4 and IL-5 concentrations in NEC serum samples compared to healthy controls.[49] Although we explored a set of twenty different cytokines, we may have missed additional and important cytokines involved

in NEC onset/development. Indeed recently it was shown that transgenic IL-37 may prevent dysregulation of adaptive immunity in murine NEC, and that this cytokine modulates immune homeostasis.[72]

We acknowledge as this a single center site proof-of-concept study with a limited number of individuals (and longitudinal samples) this is a limitation. A larger multi-center study, with e.g. a greater divergence in clinical care regimens, may allow additional key differences to be teased apart, but this was not possible in our limited single center study. Furthermore, samples were sequenced in 2014 and could not be re-sequenced due to a lack of sufficient material, which may have impacted our microbiota data. Shotgun metagenomic sequencing could provide more specific results including at the species and functional level thus providing a more comprehensive overview of microbiota changes prior to NEC onset. Moreover, relative stool hydration could have influenced the protein content in faecal samples and thus, affected overall cytokine measurements. For this reason, standardization of input material before subjection to cytokine measurement may enhance robustness and accuracy in further studies.

## Conclusion

These findings suggest that faecal cytokine concentrations could provide additional measures in the diagnosis of NEC. Particularly IL-1α, IL-10 and IL-5, which show a rise from healthy to NEC 1 to NEC2/3 and could potentially be used as accessory markers to the current Bell staging that is routinely performed. The timing of sampling and a rapid analysis yielding results within 24h would be essential for the most effective use of faecal cytokine measurement in aiding the diagnosis of NEC. Our data indicates that profiling faecal cytokine levels, particularly IL-5 and IL-10, from 14 days onwards, and

regular testing every third day for increasing levels could act as a predictive test, warning of developing NEC, but this needs to be confirmed in a larger, multi-center study. However, robust reference values of healthy preterm infants and other NEC cases from other NICUs will be required to define highly selective and sensitive cytokine thresholds, in order to provide additional information and guidance to neonatologists in the diagnosis of NEC. Additional research will also need to test and validate different platforms for faecal cytokine analysis, and compare different preterm infant cohorts to explore cytokine profile variation across different NICUs as a robust markers would be key for next stage studies. Although further testing is required, development of an early diagnosis could refine therapeutic measures, mitigate disease outcomes, increase survival rates and reduce long-term consequences for survivors.

## Figure Legends

***Figure1.*** *A: Alpha-diversity shown as richness. B: Inter-sample differences shown as multi-dimensional scaling plots based on generalized Unifrac distances across all samples and separated by different time points. C: Taxonomic differences across all samples (Bifidobacterium) and at time point 4. Numbers in brackets indicate the number of samples positive for the observation. D: Over time age matched taxonomic profiles at the phylum level of preterm babies that developed NEC (left) compared to healthy individuals (right). The DOL of NEC diagnosis is indicated after NEC samples. P-value summary: *<0.05; **<0.01.*

***Figure2.*** *Cytokine levels measured in faecal samples of preterm infants in the three study groups: Bell stage 1 NEC, Bell stage 2/3 NEC, and healthy. A: Across all time*

*points. B: Divided by time points for IL-1α, IL-5, and IL-10. At TP3, as only one sample was present in the NEC 1 group, it was excluded from the analysis. Concentrations in pg/g are plotted on a log 10 scale for better visibility. C: Significant cytokines 5-10 days before NEC diagnosis compared to age matched controls. D: Significant cytokines 11-17 days before NEC diagnosis compared to age matched controls. Numbers in brackets indicate the number of samples (one per individual) positive for the observation (if NA was reported, the number of samples is reduced). Comparisons for panels A and B were statistically analysed with Mann-Whitney-U Test. Comparisons for panels C and D were statistically analysed with Wilcoxon Rank Sum Test. P-value summary: *<0.05; **<0.01; ***<0.001; ****<0.0001.*

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## References:


1. WorldHealthOrganization. International statistical classification of diseases and related health problems. 2015;
2. Alsaied A, Islam N, Thalib L. Global incidence of Necrotizing Enterocolitis: a systematic review and Meta-analysis. *BMC pediatrics*. 2020;20(1):344. doi:10.1186/s12887-020-02231-5 Accessed 2020/07//.
3. Mekonnen SM, Bekele DM, Fenta FA, Wake AD. The Prevalence of Necrotizing Enterocolitis and Associated Factors Among Enteral Fed Preterm and Low Birth Weight Neonates Admitted in Selected Public Hospitals in Addis Ababa, Ethiopia: A Cross-sectional Study. *Global Pediatric Health*. 2021/01/01 2021;8:2333794X211019695. doi:10.1177/2333794X211019695
4. Isayama T, Lee SK, Mori R, Kusuda S, Fujimura M, Ye XY, Shah PS, the Canadian Neonatal N, the Neonatal Research Network of J. Comparison of Mortality and Morbidity of Very Low Birth Weight Infants Between Canada and Japan. *Pediatrics*. 2012;130(4):e957-e965. doi:10.1542/peds.2012-0336
5. Dammermann HJ. The clinical picture of necrotizing enterocolitis. *Arztl Wochensch*. Feb 28 1947;1-2(31-32):481-6. Das Krankheitsbild der nekrotisierenden Enterocolitis.
6. Schnabl KL, Van Aerde JE, Thomson AB, Clandinin MT. Necrotizing enterocolitis: a multifactorial disease with no cure. *World J Gastroenterol*. Apr 14 2008;14(14):2142-61. doi:10.3748/wjg.14.2142
7. Luig M, Lui K. Epidemiology of necrotizing enterocolitis--Part II: Risks and susceptibility of premature infants during the surfactant era: a regional study. *J Paediatr Child Health*. Apr 2005;41(4):174-9. doi:10.1111/j.1440-1754.2005.00583.x
8. Samuels N, van de Graaf RA, de Jonge RCJ, Reiss IKM, Vermeulen MJ. Risk factors for necrotizing enterocolitis in neonates: a systematic review of prognostic studies. *BMC Pediatr*. Apr 14 2017;17(1):105. doi:10.1186/s12887-017-0847-3
9. Quigley M, Embleton ND, McGuire W. Formula versus donor breast milk for feeding preterm or low birth weight infants. *Cochrane Database Syst Rev*. Jun 20 2018;6(6):Cd002971. doi:10.1002/14651858.CD002971.pub4
10. Berkhout DJC, Klaassen P, Niemarkt HJ, de Boode WP, Cossey V, van Goudoever JB, Hulzebos CV, Andriessen P, van Kaam AH, Kramer BW, van Lingen RA, Vijlbrief DC, van Weissenbruch MM, Benninga M, de Boer NKH, de Meij TGJ. Risk Factors for Necrotizing Enterocolitis: A Prospective Multicenter Case-Control Study. *Neonatology*. 2018;114(3):277-284. doi:10.1159/000489677
11. Wang Y, Hoenig JD, Malin KJ, Qamar S, Petrof EO, Sun J, Antonopoulos DA, Chang EB, Claud EC. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. *Isme j*. Aug 2009;3(8):944-54. doi:10.1038/ismej.2009.37
12. Pammi M, Cope J, Tarr PI, Warner BB, Morrow AL, Mai V, Gregory KE, Kroll JS, McMurtry V, Ferris MJ, Engstrand L, Lilja HE, Hollister EB, Versalovic J, Neu J. Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis. *Microbiome*. Mar 9 2017;5(1):31. doi:10.1186/s40168-017-0248-8
13. Sim K, Shaw AG, Randell P, Cox MJ, McClure ZE, Li MS, Haddad M, Langford PR, Cookson WO, Moffatt MF, Kroll JS. Dysbiosis anticipating necrotizing enterocolitis in very premature infants. *Clin Infect Dis*. Feb 1 2015;60(3):389-97. doi:10.1093/cid/ciu822
14. Reyman M, van Houten MA, van Baarle D, Bosch AATM, Man WH, Chu MLJN, Arp K, Watson RL, Sanders EAM, Fuentes S, Bogaert D. Impact of delivery mode-associated gut microbiota dynamics on health in the first year of life. *Nature Communications*. 2019/11/01 2019;10(1):4997. doi:10.1038/s41467-019-13014-7
15. Shao Y, Forster SC, Tsaliki E, Vervier K, Strang A, Simpson N, Kumar N, Stares MD, Rodger A, Brocklehurst P, Field N, Lawley TD. Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. *Nature*. Oct 2019;574(7776):117-121. doi:10.1038/s41586-019-1560-1
16. Coggins SA, Wynn JL, Weitkamp J-H. Infectious causes of necrotizing enterocolitis. *Clinics in perinatology*. 2015;42(1):133-ix. doi:10.1016/j.clp.2014.10.012

17. Alcon-Giner C, Dalby MJ, Caim S, Ketskemety J, Shaw A, Sim K, Lawson MAE, Kiu R, Leclaire C, Chalklen L, Kujawska M, Mitra S, Fardus-Reid F, Belteki G, McColl K, Swann JR, Kroll JS, Clarke P, Hall LJ. Microbiota Supplementation with Bifidobacterium and Lactobacillus Modifies the Preterm Infant Gut Microbiota and Metabolome: An Observational Study. *Cell Rep Med*. Aug 25 2020;1(5):100077. doi:10.1016/j.xcrm.2020.100077

18. Robertson C, Savva GM, Clapuci R, Jones J, Maimouni H, Brown E, Minocha A, Hall LJ, Clarke P. Incidence of necrotising enterocolitis before and after introducing routine prophylactic Lactobacillus and Bifidobacterium probiotics. *Arch Dis Child Fetal Neonatal Ed*. Jul 2020;105(4):380-386. doi:10.1136/archdischild-2019-317346

19. Bell M, Cole CR, Hansen NI, Duncan AF, Hintz SR, Adams-Chapman I. Neurodevelopmental and Growth Outcomes of Extremely Preterm Infants with Short Bowel Syndrome. *J Pediatr*. Mar 2021;230:76-83.e5. doi:10.1016/j.jpeds.2020.11.026

20. Shin SH, Kim EK, Kim SH, Kim HY, Kim HS. Head Growth and Neurodevelopment of Preterm Infants with Surgical Necrotizing Enterocolitis and Spontaneous Intestinal Perforation. *Children (Basel)*. Sep 23 2021;8(10)doi:10.3390/children8100833

21. Cha JH, Lim JS, Jang YH, Hwang JK, Na JY, Lee JM, Lee HJ, Ahn JH. Altered microstructure of the splenium of corpus callosum is associated with neurodevelopmental impairment in preterm infants with necrotizing enterocolitis. *Ital J Pediatr*. Jan 10 2022;48(1):6. doi:10.1186/s13052-021-01197-z

22. Bell MJ. Neonatal necrotizing enterocolitis. *N Engl J Med*. Feb 2 1978;298(5):281-2.

23. Walsh MC, Kliegman RM. Necrotizing enterocolitis: treatment based on staging criteria. *Pediatr Clin North Am*. Feb 1986;33(1):179-201. doi:10.1016/s0031-3955(16)34975-6

24. Patel RM, Ferguson J, McElroy SJ, Khashu M, Caplan MS. Defining necrotizing enterocolitis: current difficulties and future opportunities. *Pediatr Res*. Aug 2020;88(Suppl 1):10-15. doi:10.1038/s41390-020-1074-4

25. Gephart SM, Gordon PV, Penn AH, Gregory KE, Swanson JR, Maheshwari A, Sylvester K. Changing the paradigm of defining, detecting, and diagnosing NEC: Perspectives on Bell's stages and biomarkers for NEC. *Semin Pediatr Surg*. Feb 2018;27(1):3-10. doi:10.1053/j.sempedsurg.2017.11.002

26. Dobbler PT, Procianoy RS, Mai V, Silveira RC, Corso AL, Rojas BS, Roesch LFW. Low Microbial Diversity and Abnormal Microbial Succession Is Associated with Necrotizing Enterocolitis in Preterm Infants. *Front Microbiol*. 2017;8:2243. doi:10.3389/fmicb.2017.02243

27. Morrow AL, Lagomarcino AJ, Schibler KR, Taft DH, Yu Z, Wang B, Altaye M, Wagner M, Gevers D, Ward DV, Kennedy MA, Huttenhower C, Newburg DS. Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants. *Microbiome*. 2013/04/16 2013;1(1):13. doi:10.1186/2049-2618-1-13

28. Brower-Sinning R, Zhong D, Good M, Firek B, Baker R, Sodhi CP, Hackam DJ, Morowitz MJ. Mucosa-Associated Bacterial Diversity in Necrotizing Enterocolitis. *PLoS One*. 2014;9(9):e105046. doi:10.1371/journal.pone.0105046

29. Torrazza RM, Ukhanova M, Wang X, Sharma R, Hudak ML, Neu J, Mai V. Intestinal Microbial Ecology and Environmental Factors Affecting Necrotizing Enterocolitis. *PLoS One*. 2014;8(12):e83304. doi:10.1371/journal.pone.0083304

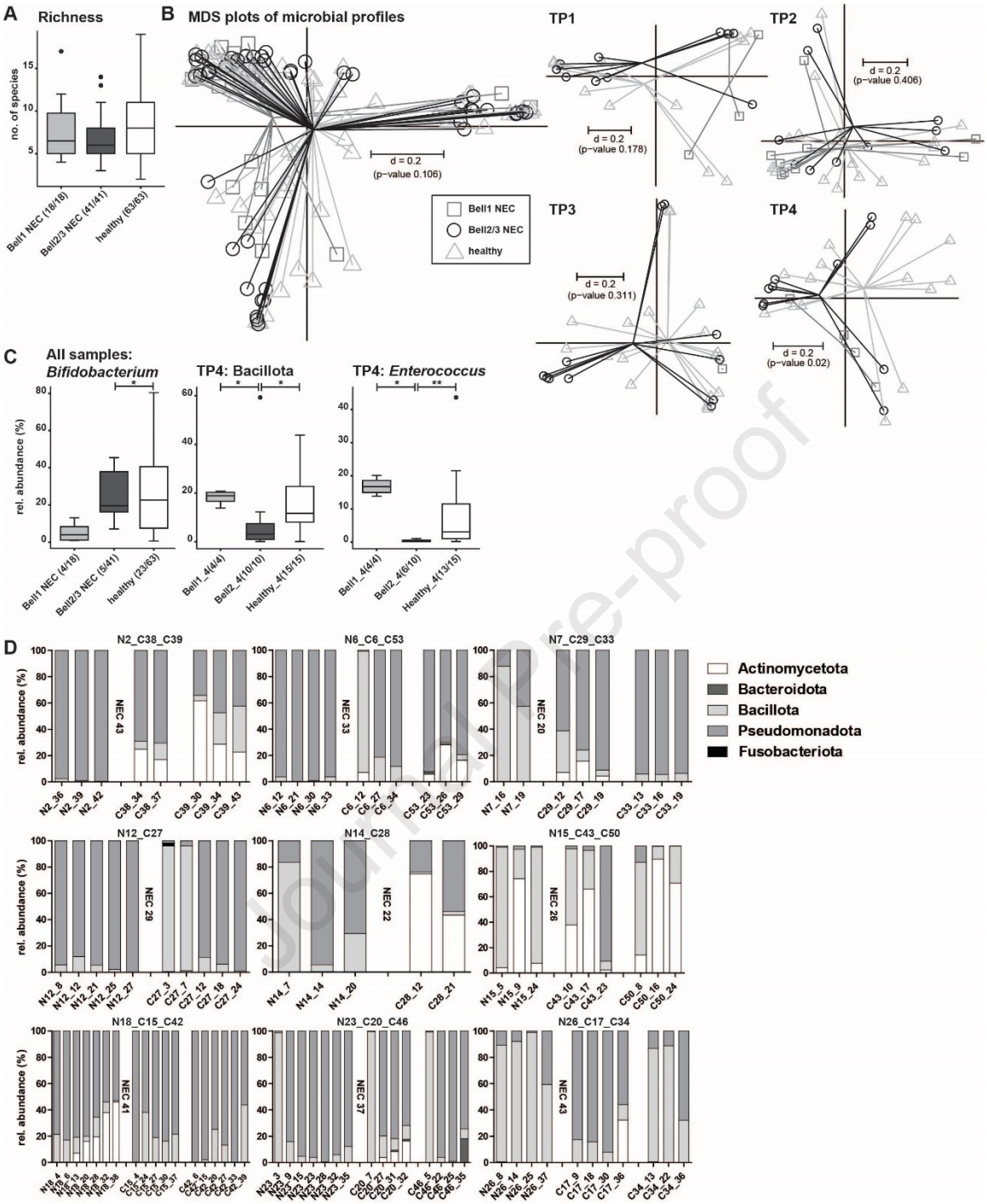
30. Ward DV, Scholz M, Zolfo M, Taft DH, Schibler KR, Tett A, Segata N, Morrow AL. Metagenomic Sequencing with Strain-Level Resolution Implicates Uropathogenic E. coli in Necrotizing Enterocolitis and Mortality in Preterm Infants. *Cell Rep*. Mar 29 2016;14(12):2912-24. doi:10.1016/j.celrep.2016.03.015

31. Warner BB, Deych E, Zhou Y, Hall-Moore C, Weinstock GM, Sodergren E, Shaikh N, Hoffmann JA, Linneman LA, Hamvas A, Khanna G, Rouggly-Nickless LC, Ndao IM, Shands BA, Escobedo M, Sullivan JE, Radmacher PG, Shannon WD, Tarr PI. Gut bacteria dysbiosis and necrotising enterocolitis in very low birthweight infants: a prospective case-control study. *Lancet*. May 7 2016;387(10031):1928-36. doi:10.1016/s0140-6736(16)00081-7

32. Gopalakrishna KP, Macadangdang BR, Rogers MB, Tometich JT, Firek BA, Baker R, Ji J, Burr AHP, Ma C, Good M, Morowitz MJ, Hand TW. Maternal IgA protects against the development of necrotizing enterocolitis in preterm infants. *Nat Med*. Jul 2019;25(7):1110-1115. doi:10.1038/s41591-019-0480-9
33. Olm MR, Bhattacharya N, Crits-Christoph A, Firek BA, Baker R, Song YS, Morowitz MJ, Banfield JF. Necrotizing enterocolitis is preceded by increased gut bacterial replication, Klebsiella, and fimbriae-encoding bacteria. *Sci Adv*. Dec 2019;5(12):eaax5727. doi:10.1126/sciadv.aax5727
34. Cassir N, Benamar S, Khalil JB, Croce O, Saint-Faust M, Jacquot A, Million M, Azza S, Armstrong N, Henry M, Jardot P, Robert C, Gire C, Lagier JC, Chabrière E, Ghigo E, Marchandin H, Sartor C, Boutte P, Cambonie G, Simeoni U, Raoult D, La Scola B. Clostridium butyricum Strains and Dysbiosis Linked to Necrotizing Enterocolitis in Preterm Neonates. *Clin Infect Dis*. Oct 1 2015;61(7):1107-15. doi:10.1093/cid/civ468
35. Yu Z-T, Chen C, Kling DE, Liu B, McCoy JM, Merighi M, Heidtman M, Newburg DS. The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota. *Glycobiology*. 2013;23(2):169-177. doi:10.1093/glycob/cws138
36. Sodhi CP, Wipf P, Yamaguchi Y, Fulton WB, Kovler M, Niño DF, Zhou Q, Banfield E, Werts AD, Ladd MR, Buck RH, Goehring KC, Prindle T, Jr., Wang S, Jia H, Lu P, Hackam DJ. The human milk oligosaccharides 2'-fucosyllactose and 6'-sialyllactose protect against the development of necrotizing enterocolitis by inhibiting toll-like receptor 4 signaling. *Pediatr Res*. Jan 2021;89(1):91-101. doi:10.1038/s41390-020-0852-3
37. Masi AC, Embleton ND, Lamb CA, Young G, Granger CL, Najera J, Smith DP, Hoffman KL, Petrosino JF, Bode L, Berrington JE, Stewart CJ. Human milk oligosaccharide DSLNT and gut microbiome in preterm infants predicts necrotising enterocolitis. *Gut*. Dec 2021;70(12):2273-2282. doi:10.1136/gutjnl-2020-322771
38. Cho SX, Berger PJ, Nold-Petry CA, Nold MF. The immunological landscape in necrotising enterocolitis. *Expert Rev Mol Med*. Jun 24 2016;18:e12. doi:10.1017/erm.2016.13
39. Kim JH, Sampath V, Canvasser J. Challenges in diagnosing necrotizing enterocolitis. *Pediatr Res*. Aug 2020;88(Suppl 1):16-20. doi:10.1038/s41390-020-1090-4
40. Kenton AB, O'Donovan D, Cass DL, Helmrath MA, Smith EO, Fernandes CJ, Washburn K, Weihe EK, Brandt ML. Severe thrombocytopenia predicts outcome in neonates with necrotizing enterocolitis. *J Perinatol*. Jan 2005;25(1):14-20. doi:10.1038/sj.jp.7211180
41. Duci M, Fascetti-Leon F, Erculiani M, Priante E, Cavicchiolo ME, Verlato G, Gamba P. Neonatal independent predictors of severe NEC. *Pediatr Surg Int*. Jun 2018;34(6):663-669. doi:10.1007/s00383-018-4261-1
42. Çetinkaya M, Özkan H, Köksal N, Akacı O, Özgür T. The efficacy of serial serum amyloid A measurements for diagnosis and follow-up of necrotizing enterocolitis in premature infants. *Pediatric Surgery International*. 2010/08/01 2010;26(8):835-841. doi:10.1007/s00383-010-2635-0
43. Bein A, Eventov-Friedman S, Arbell D, Schwartz B. Intestinal tight junctions are severely altered in NEC preterm neonates. *Pediatr Neonatol*. Oct 2018;59(5):464-473. doi:10.1016/j.pedneo.2017.11.018
44. Feenstra FA, Kuik SJ, Derikx JPM, Heiner-Fokkema MR, Kooi EMW, Bos AF, Hulscher JBF. Plasma citrulline during the first 48 h after onset of necrotizing enterocolitis in preterm infants. *J Pediatr Surg*. Mar 2021;56(3):476-482. doi:10.1016/j.jpedsurg.2020.11.020
45. Jawale N, Prideaux M, Prasad M, Miller M, Rastogi S. Plasma Citrulline as a Biomarker for Early Diagnosis of Necrotizing Enterocolitis in Preterm Infants. *Am J Perinatol*. Nov 2021;38(13):1435-1441. doi:10.1055/s-0040-1713406
46. Xu Y, Liu Y, Xie H, Zhou Y, Yan X, Chen W, Wang X, Yu Z, Wang F, Chen X, Wang J, Han S. Profile analysis reveals endogenous RNAs regulate necrotizing enterocolitis progression. *Biomed Pharmacother*. May 2020;125:109975. doi:10.1016/j.biopha.2020.109975
47. Wright H, Bannaga AS, Iriarte R, Mahmoud M, Arasaradnam RP. Utility of volatile organic compounds as a diagnostic tool in preterm infants. *Pediatr Res*. Jan 2021;89(2):263-268. doi:10.1038/s41390-020-0828-3

48. Thibault MP, Tremblay É, Horth C, Fournier-Morin A, Grynspan D, Babakissa C, Levy E, Ferretti E, Bertelle V, Beaulieu JF. Lipocalin-2 and calprotectin as stool biomarkers for predicting necrotizing enterocolitis in premature neonates. *Pediatr Res*. Jan 2022;91(1):129-136. doi:10.1038/s41390-021-01680-7
49. Benkoe T, Baumann S, Weninger M, Pones M, Reck C, Rebhandl W, Oehler R. Comprehensive Evaluation of 11 Cytokines in Premature Infants with Surgical Necrotizing Enterocolitis. *PLoS One*. 2013;8(3):e58720. doi:10.1371/journal.pone.0058720
50. Wisgrill L, Weinhandl A, Unterasinger L, Amann G, Oehler R, Metzelder ML, Berger A, Benkoe TM. Interleukin-6 serum levels predict surgical intervention in infants with necrotizing enterocolitis. *J Pediatr Surg*. Mar 2019;54(3):449-454. doi:10.1016/j.jpedsurg.2018.08.003
51. Yin Y, Wu X, Zou H, Li S, Qin Z, Zhang T, Cao J. Expressions of Fib, IL-12 in Serum of Neonatal Necrotizing Enterocolitis and Their Correlation with Clinicopathological Features. *Iran J Public Health*. Aug 2020;49(8):1439-1446. doi:10.18502/ijph.v49i8.3874
52. Seo YM, Lin YK, Im SA, Sung IK, Youn YA. Interleukin 8 may predict surgical necrotizing enterocolitis in infants born less than 1500 g. *Cytokine*. Jan 2021;137:155343. doi:10.1016/j.cyto.2020.155343
53. Delanghe JR, Speeckaert MM. Translational research and biomarkers in neonatal sepsis. *Clin Chim Acta*. Dec 7 2015;451(Pt A):46-64. doi:10.1016/j.cca.2015.01.031
54. Cnattingius S, Johansson S, Razaz N. Apgar Score and Risk of Neonatal Death among Preterm Infants. *N Engl J Med*. Jul 2 2020;383(1):49-57. doi:10.1056/NEJMoa1915075
55. Dahlgren AF, Pan A, Lam V, Gouthro KC, Simpson PM, Salzman NH, Nghiem-Rao TH. Longitudinal changes in the gut microbiome of infants on total parenteral nutrition. *Pediatr Res*. Jul 2019;86(1):107-114. doi:10.1038/s41390-019-0391-y
56. Arboleya S, Binetti A, Salazar N, Fernández N, Solís G, Hernández-Barranco A, Margolles A, de Los Reyes-Gavilán CG, Gueimonde M. Establishment and development of intestinal microbiota in preterm neonates. *FEMS Microbiol Ecol*. Mar 2012;79(3):763-72. doi:10.1111/j.1574-6941.2011.01261.x
57. Cong X, Xu W, Janton S, Henderson WA, Matson A, McGrath JM, Maas K, Graf J. Gut Microbiome Developmental Patterns in Early Life of Preterm Infants: Impacts of Feeding and Gender. *PLoS One*. 2016;11(4):e0152751. doi:10.1371/journal.pone.0152751
58. Wandro S, Osborne S, Enriquez C, Bixby C, Arrieta A, Whiteson K. The Microbiome and Metabolome of Preterm Infant Stool Are Personalized and Not Driven by Health Outcomes, Including Necrotizing Enterocolitis and Late-Onset Sepsis. *mSphere*. Jun 27 2018;3(3)doi:10.1128/mSphere.00104-18
59. Claud EC, Walker WA. Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. *Faseb j*. Jun 2001;15(8):1398-403. doi:10.1096/fj.00-0833hyp
60. Warner BB, Tarr PI. Necrotizing enterocolitis and preterm infant gut bacteria. *Semin Fetal Neonatal Med*. Dec 2016;21(6):394-399. doi:10.1016/j.siny.2016.06.001
61. Abellan-Schneyder I, Matchado MS, Reitmeier S, Sommer A, Sewald Z, Baumbach J, List M, Neuhaus K. Primer, Pipelines, Parameters: Issues in 16S rRNA Gene Sequencing. *mSphere*. Feb 24 2021;6(1)doi:10.1128/mSphere.01202-20
62. Johnson JS, Spakowicz DJ, Hong B-Y, Petersen LM, Demkowicz P, Chen L, Leopold SR, Hanson BM, Agresta HO, Gerstein M, Sodergren E, Weinstock GM. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nature Communications*. 2019/11/06 2019;10(1):5029. doi:10.1038/s41467-019-13036-1
63. Zanella A, Silveira RC, Roesch LFW, Corso AL, Dobbler PT, Mai V, Procianoy RS. Influence of own mother's milk and different proportions of formula on intestinal microbiota of very preterm newborns. *PLoS One*. 2019;14(5):e0217296. doi:10.1371/journal.pone.0217296
64. Schirmer M, Smeekens SP, Vlamakis H, Jaeger M, Oosting M, Franzosa EA, Ter Horst R, Jansen T, Jacobs L, Bonder MJ, Kurilshikov A, Fu J, Joosten LAB, Zhernakova A, Huttenhower C, Wijmenga C,

577 Netea MG, Xavier RJ. Linking the Human Gut Microbiome to Inflammatory Cytokine Production
578 Capacity. *Cell*. 2016;167(4):1125-1136.e8. doi:10.1016/j.cell.2016.10.020
579 65. Dinarello CA, Simon A, van der Meer JW. Treating inflammation by blocking interleukin-1 in a
580 broad spectrum of diseases. *Nat Rev Drug Discov*. Aug 2012;11(8):633-52. doi:10.1038/nrd3800
581 66. Ng PC, Li K, Wong RPO, Chui K, Wong E, Li G, Fok TF. Proinflammatory and anti-inflammatory
582 cytokine responses in preterm infants with systemic infections. *Archives of disease in childhood Fetal
583 and neonatal edition*. 2003;88(3):F209-F213. doi:10.1136/fn.88.3.f209
584 67. Garofalo R, Chheda S, Mei F, Palkowetz KH, Rudloff HE, Schmalstieg FC, Rassin DK, Goldman
585 AS. Interleukin-10 in Human Milk. *Pediatric Research*. 1995/04/01 1995;37(4):444-449.
586 doi:10.1203/00006450-199504000-00010
587 68. Fituch CC, Palkowetz KH, Goldman AS, Schanler RJ. Concentrations of IL-10 in preterm human
588 milk and in milk from mothers of infants with necrotizing enterocolitis. *Acta Paediatr*. Nov
589 2004;93(11):1496-500. doi:10.1080/08035250410022314
590 69. Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E, Klunker S, Meyer N, O'Mahony
591 L, Palomares O, Rhyner C, Ouaked N, Schaffartzik A, Van De Veen W, Zeller S, Zimmermann M, Akdis
592 CA. Interleukins, from 1 to 37, and interferon-γ: receptors, functions, and roles in diseases. *J Allergy
593 Clin Immunol*. Mar 2011;127(3):701-21.e1-70. doi:10.1016/j.jaci.2010.11.050
594 70. Calhoun DA, Sullivan SE, Lunøe M, Du Y, Christensen RD. Granulocyte-macrophage colony-
595 stimulating factor and interleukin-5 concentrations in premature neonates with eosinophilia. *J
596 Perinatol*. Apr-May 2000;20(3):166-71. doi:10.1038/sj.jp.7200332
597 71. Liu Y, Zhu L, Fatheree NY, Liu X, Pacheco SE, Tatevian N, Rhoads JM. Changes in intestinal
598 Toll-like receptors and cytokines precede histological injury in a rat model of necrotizing
599 enterocolitis. *American journal of physiology Gastrointestinal and liver physiology*. 2009;297(3):G442-
600 G450. doi:10.1152/ajpgi.00182.2009
601 72. Cho SX, Rudloff I, Lao JC, Pang MA, Goldberg R, Bui CB, McLean CA, Stock M, Klassert TE,
602 Slevogt H, Mangan NE, Cheng W, Fischer D, Gfroerer S, Sandhu MK, Ngo D, Bujotzek A, Lariviere L,
603 Schumacher F, Tiefenthaler G, Beker F, Collins C, Kamlin COF, König K, Malhotra A, Tan K, Theda C,
604 Veldman A, Ellisdon AM, Whisstock JC, Berger PJ, Nold-Petry CA, Nold MF. Characterization of the
605 pathoimmunology of necrotizing enterocolitis reveals novel therapeutic opportunities. *Nat Commun*.
606 Nov 13 2020;11(1):5794. doi:10.1038/s41467-020-19400-w
607 73. Lagkouvardos I, Joseph D, Kapfhammer M, Giritli S, Horn M, Haller D, Clavel T. IMNGS: A
608 comprehensive open resource of processed 16S rRNA microbial profiles for ecology and diversity
609 studies. *Scientific Reports*. 2016/09/23 2016;6(1):33721. doi:10.1038/srep33721
610 74. Lagkouvardos I, Fischer S, Kumar N, Clavel T. Rhea: a transparent and modular R pipeline for
611 microbial profiling based on 16S rRNA gene amplicons. *PeerJ*. 2017/01/11 2017;5:e2836.
612 doi:10.7717/peerj.2836


A
Richness
no. of species
Bell1 NEC (18/18)
Bell2/3 NEC (41/41)
healthy (63/63)
B
MDS plots of microbial profiles
d = 0.2
(p−value 0.106)
Bell1 NEC
Bell2/3 NEC
healthy
TP1
d = 0.2
(p−value 0.178)
TP2
d = 0.2
(p−value 0.406)
TP3
d = 0.2
(p−value 0.311)
TP4
d = 0.2
(p−value 0.02)
C
All samples:
Bifidobacterium
rel. abundance (%)
Bell1 NEC (4/18)
Bell2/3 NEC (5/41)
healthy (23/63)
TP4: Bacillota
Bell1_4(4/4)
Bell2_4(10/10)
Healthy_4(15/15)
TP4: Enterococcus
Bell1_4(4/4)
Bell2_4(6/10)
Healthy_4(13/15)
D
N2_C38_C39
N6_C6_C53
N7_C29_C33
N12_C27
N14_C28
N15_C43_C50
N18_C15_C42
N23_C20_C46
N26_C17_C34
rel. abundance (%)
Actinomycetota
Bacteroidota
Bacillota
Pseudomonadota
Fusobacteriota