Study Title: Alteration of Stool Microbiota in Preterm Infants Less than 32 Weeks with Anemia, and Following Blood Transfusion

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Study Title:
Alteration of Stool Microbiota in Preterm Infants Less than 32 Weeks with Anemia, and Following Blood Transfusion

Investigators:
Kshiti Shah, MD
Julie Mirpuri, MD (PI)

Type of Funding:
Institutional

Purpose:
- To determine if the stool microbiota in preterm infants is altered with anemia.
- To determine if the stool microbiota in preterm infants is altered after blood transfusion.

Background:
Neonatal gut microbiota begins developing at approximately 2 weeks of age and is less diverse than the microbiota of a term infant. Four bacterial classes make up more than 90% of the microbes present in the neonate: *Bacilli, Gammaproteobacteria, Clostridia, and Negativicutes.* There are multiple factors that influence the neonatal gut microbiota, including mode of infant delivery, type of feeds (i.e. formula or breast milk), medications such as antibiotics, and exposure to pathogens in a hospital environment.

Dysbiosis occurs when there is an imbalance in the proportion of bacterial pathogens and commensal bacteria. This can cause a shift away from homeostasis and lead to an inflammatory state and disease. Studies utilizing molecular methods suggest that dysbiosis occurs in neonates with necrotizing enterocolitis (NEC). While results vary, large studies and meta-analyses suggest a predominance of *Gammaproteobacteria* with NEC and a decrease in *Firmicutes* with NEC. There may also be a decrease in bacterial diversity prior to NEC onset.

The relationship between transfusion and necrotizing enterocolitis has been controversial and frequently studied. Despite this, there is a lack of good quality studies on this topic with conflicted findings and the literature does not currently support a relationship between transfusions and NEC. There have been limited studies on the relationship between anemia and NEC. Some suggest that severe anemia and NEC are associated, however this has not been shown in other studies.

While the evidence seems to support a relationship between a change in microbiota and NEC, there is no clear relationship between anemia or transfusion and NEC. There have been no studies evaluating the relationship between anemia or transfusion and a change in microbiota. That is the primary focus of this study.

Hypothesis:
Anemia will result in alteration of the stool microbiota. Transfusion will return microbiota to baseline. The specific alteration in microbiota with anemia may include: increase in *Proteobacteria*, decrease in *Firmicutes*, and/or decrease in microbiota diversity.

Primary Outcome:
- Change in microbiota with anemia

Secondary Outcome:
- Change in microbiota after transfusion

Inclusion Criteria:
- Preterm infants less than 32 weeks gestation at birth
- $\geq$ 7 days and <30 days on enrollment
- On minimum 100mL/kg enteral feeds

Exclusion Criteria:
- NEC prior to enrollment
- Prior surgery
- Major congenital anomalies
- Oxygen requirement with FiO2 >50%

Methods:

Definitions:
- Severe anemia will be defined as a hematocrit < 25%.
- Anemia will be defined as a hematocrit $\geq$ 25 and <30%.
- No anemia will be defined as a hematocrit $\geq$ 30%.

Hematocrit will be tested per patient's care team. In this unit, hematocrit is usually obtained every Monday, and sometimes more frequently.

Sample Collection:
- Collect stool samples weekly (Sunday, Monday, or Tuesday)
- Collect stool samples 1, 4, and 7 days post-transfusion
- Stool will continue to be collected until infants are 38 weeks corrected gestational age (CGA) or discharge (whichever is earlier).

Data Collection:

Hematocrit	Gestational Age
Birthweight	Apgars
Sex	Race
Singleton/Multiple	Age at stool collection and at hematocrit blood sampling
Iron supplementation	Type of feeds (breastmilk/formula feed)
Calorie fortification of feeds	Total days on antibiotics
Number of transfusions	Positive cultures
NEC	Indomethacin exposure

Groups:
Paired samples will be obtained from the same infants, one before and one after onset of anemia. Therefore, the patients will serve as their own controls.

Sample Analysis:
- Stool samples obtained while patient is on antibiotics will not be included in analysis since the microbiota is expected to be significantly changed with large populations wiped out. Stool samples obtained 5 days after completion of antibiotics will be included in analysis again since infants are expected to be recolonized by that time.
- qRT-PCR of samples will be performed to quantify the major bacterial groups: *Proteobacteria, Firmicutes,* and *Bacteroides.*
- Next generation sequencing will be done for qualitative analysis and metagenomics profiling of bacteria

Informed Consent:
New admissions to the Parkland NICU will be screened for recruitment if their birth gestational age (OB assigned) is less than 32 weeks 0 days and if they are at least 7 days old.

• If they meet these two criteria, they will be screened to ensure they do not meet exclusion criteria.

• If they do not meet the exclusion criteria, then their feeds will be evaluated. If they are on at least 100mL/kg/day of enteral feeds, then they will be approached for recruitment. Infants that are not yet on 100mL/kg/day of enteral feeds will have their charts assessed daily until 29 days of life. If and when they achieve 100mL/kg/day of enteral feeds, they will be approached for recruitment. If an infant does not achieve 100mL/kg/day of enteral feeds before 30 days of life, they will not be approached for enrollment in this study.
• If an infant meets the above criteria, and once their parent/guardian/LAR provides consent, then stool collection will begin for that infant from the next available defecated diaper. In cases where the patients are not the PI's patients, the study team will inform all the physicians caring for the patients within the NICU about this research study and

once they approve for their patients' (infant's) parents/guardians/LARs to be approached for consenting purpose to enroll the infant into this study, the bedside nurse will check with the parents/guardians/LARs if they would like to be approached. Once they let the beside nurse know if they agree to be approached, the nurse would inform this to Dr. Mirpuri (the PI) and Dr. Shah (study team) who will be responsible for recruitment of subjects and they would approach the patient's parent/guardian/LAR for consenting purposes.

Sample Size Calculation:
Group sample sizes of 194 achieve 80% power to detect an odds ratio of 1.500 in a design with 2 repeated measurements having a simple covariance structure when the proportion for group 2 is 0.4 and group 1 is 0.5. The alpha level is 0.05.

Statistical Analysis:
Descriptive analysis will use percentages, means, medians, and measures of variability to describe demographic data. Categorical factors will be evaluated using Chi-square or Fisher exact test between the groups. Continuous factors will be evaluated using Student's t-test or Mann-Whitney U test if the data is non-normal distributed between the groups. Repeated measurements will use analysis of variance (ANOVA) to compare groups. The statistical significance level will be 0.05 for all statistical tests.