A single-center, masked, randomized, superiority trial of early life protein-enriched human milk diets to increase lean body mass accretion and diversity of the gut microbiome in extremely preterm infants

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SECTION 1. ABSTRACT

Study Hypothesis/Question
The central hypothesis of the proposed work is that protein-enriched human milk diets during the first 2 weeks after birth increase fat-free mass(FFM)-for-age Z scores and promote maturation of the gut microbiome at term equivalent age in extremely preterm (EPT) infants.

Study Design Type
Parallel-group, masked randomized controlled trial in which study participants fed human milk will be randomly assigned in a 1:1 allocation ratio to receive either a protein-enriched human milk diet (intervention group) or a usual human milk diet (control group) within the first 96 hours after birth..

Eligibility Criteria
EPT infants with gestational age of 28 weeks or less admitted to the neonatal unit at the University of Alabama at Birmingham (UAB) Hospital will be included. Infants with major congenital anomalies and infants with a terminal illness in whom decisions to withhold or limit life support have been made will be excluded.

Study Intervention/Methods
Written informed consent will be obtained within the first 96 hours after birth to allow treatment allocation before or on the first day of enteral feeding via orogastric tube. Infants in the intervention group will receive expressed human milk or donor human milk on feeding day 1 (within the first 96 hours after birth). On feeding day 2, a human milk-based product that increases protein content (Prolact®, Prolacta Bioscience, Inc. City of Industry, CA) will be added to human milk. This practice will continue until standard bovine-based human milk fortifiers are ordered. Infants in the control group will receive expressed human milk or donor human milk from feeding day 1. This practice will continue until standard bovine-based products are ordered.

Primary Outcome
The primary efficacy outcome will be FFM-for-age Z-score at 36 weeks of postmenstrual age (PMA). The primary microbiological outcome will be composition and diversity of the gut microbiome.

Secondary Outcome(s)
Secondary efficacy outcomes will include postnatal growth failure (PGF), FFM, %FFM, body fat (BF), and %BF at 36 weeks PMA, growth velocity rate (g/kg/d) between birth and 36 weeks PMA, and anthropometric measurements at 36 weeks PMA (weight, head circumference, and length). The primary safety outcomes will include intestinal perforation, NEC stage 2 or greater, culture-proven sepsis, and death.

SECTION 2. STATEMENT OF PROBLEM

2.1. PRIMARY HYPOTHESIS OR QUESTION

Protein-enriched human milk diets during the first 2 weeks after birth increase FFM-for-age Z scores at term equivalent age in extremely preterm (EPT) infants.

2.2. SECONDARY HYPOTHESIS OR QUESTIONS (S) (IF APPLICABLE)

Protein-enriched human milk diets during the first 2 weeks after birth promote maturation of the gut microbiome at term equivalent age in extremely preterm (EPT) infants.

2.3. BACKGROUND AND RATIONALE

Limited enteral nutrition aggravates the problem of cumulative nutritional deficits during the first 2 weeks after birth and increases the risk of postnatal growth failure in extremely preterm (EPT) infants born at 28 weeks of gestation or less. Postnatal growth failure occurs in approximately 6 of every 10 EPT infants by the time they reach 36 weeks of postmenstrual age (PMA). EPT infants with postnatal growth failure have a higher risk of adverse health outcomes, particularly when they have more fat mass (FM) gains than fat-free mass (FFM) gains. To restore cumulative nutritional deficits and prevent postnatal growth failure in EPT infants with limited enteral nutrition during the first 2 weeks after birth, most clinicians prescribe protein-enriched diets to promote catch-up growth only after full enteral nutrition is established. Emerging clinical evidence suggests that this approach is not effective to improve health outcomes in EPT infants.

Likewise, increasing evidence from translational studies suggest that the practice of limiting enteral nutrition in early postnatal life shapes not only growth and FFM accretion, but also development of the gastrointestinal (GI) tract and composition of the gut microbiome. EPT infants unable to receive sufficient enteral nutrition during the first 2 weeks after birth need innovative early life dietary interventions. Not addressing this problem will perpetuate the practice of limiting enteral nutrition in EPT infants during a critical period of development in which human milk diets could influence development of the GI tract and help define composition of the gut microbiome.

SECTION 3. METHODS

3.1. STUDY POPULATION

Extremely preterm infants with gestational ages of 22 0/7 to 26 6/7 weeks of gestation admitted to the UAB hospital. This study population has been selected based on the frequency of feeding problems observed at these lower gestational ages and the increased risk of postnatal growth failure in this vulnerable population.

3.1.1. Inclusion Criteria

- Gestational age of 22 0/7 to 26 6/7 weeks of gestation
- < 48 hours postnatal age

3.1.2. Exclusion Criteria

- Major congenital/chromosomal anomalies
- Terminal illness requiring limited or withheld support

3.2. DETAILED STUDY PROCEDURES

3.2.1. Screening

All 22 0/7 to 26 6/7 weeks of gestation infants admitted to the UAB neonatal unit will be screened to determine eligibility for the trial. To maximize the generalizability of our results, we will screen inborn and outborn infants in their first 96 hours to allow adequate time for informed consent, identify terminally ill infants, and exclude early deaths unrelated to enteral feeding.

3.2.2. Consent Procedures

Written informed consent will be obtained by the first 96 hours after birth to allow treatment allocation before or on the first day of enteral feeding. If a potential participant is identified, a member of the study will see the parents and/or mother in her room or the baby's room and explain the study. The risks and benefits will be discussed with the parents and time will be given to them to ask questions. It will be made known to them that no treatment will be withheld from their infant if they participate in the study. Randomization will define study group assignment.

3.2.3. Randomization Procedures

Participants will be randomly assigned to one of the study groups following computer-generated random-block sequences and with the use of numbered, opaque, sealed envelopes, which will be opened in sequential order only after informed consent is obtained. Twin infants will be randomized individually.

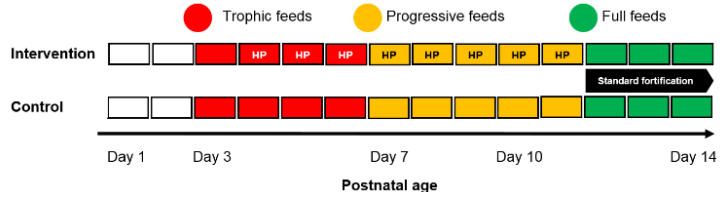
3.2.4. Study Intervention and Comparison

Usual feeding practices in our neonatal unit include: 1) administration of enteral feeds as intermittent bolus gavage every 3 hours; 2) initiation of trophic feeds within the first 96 hours after birth with 20-25 ml/kg/d; 3) progression of enteral feeds with daily increments of 20-25 ml/kg/d usually before postnatal day 7; 4) use of donor human milk as an alternative to mother's own milk if the mother is not able to supply her own milk, and 5) addition of bovine-based products that increase protein content of human milk at approximately postnatal day 14 after full enteral nutrition is established (> 120 ml/kg/d) **[Figure 4].**

Intervention group: Infants in the intervention group will receive expressed human milk or donor human milk on feeding day 1 (within the first 96 hours after birth). On feeding day 2, a human milk-based product that increases protein content (Prolact®, Prolacta Bioscience, Inc. City of Industry, CA) will be added to human milk. This practice will continue until standard bovine-based human milk fortifiers are ordered.

Control group: Infants in the control group will receive expressed human milk or donor human milk from feeding day 1. This practice will continue until standard bovine-based products are ordered.

Figure 4. Study Intervention



3.2.5. Blinding/Masking

Caregivers and primary outcome evaluators will be masked. Nutrition room staff not involved in patient care will be responsible for determining participant allocation to one of the supplementation groups by opening sequentially numbered sealed envelopes, dispensing feeding syringes with the allocated human milk diet (protein-enriched or usual), and masking caregivers administering the assigned dietary intervention.

3.2.6. Control or Monitoring of Co-interventions

This pragmatic trial will compare protein-enriched and usual human milk diets under normal clinical circumstances with no effort to strictly control interventions other than dietary intervention. Therefore, clinical care will be conducted at the clinician's discretion.

3.2.7. Primary Outcome

- The primary efficacy outcome will be FFM-for-age Z-score at 36 weeks PMA. The primary microbiological outcome will be composition and diversity of the gut microbiome

3.2.8. Secondary Outcomes

- PGF, FFM, %FFM, BF and %BF at 36 weeks PMA, growth velocity rate (g/kg/d) between birth and 36 weeks PMA, and anthropometric measures at 36 weeks PMA (weight, head circumference, and length).
- Respiratory support at 36 weeks PMA
- Bronchopulmonary dysplasia (BPD) at 36 weeks PMA

3.2.9. Additional Safety Outcomes

- Death
- NEC stage 2 or 3
- SIP
- Culture-proven sepsis

3.2.10. Compliance Monitoring

Unlike many enteral feeding trials in EPT infants, including our preliminary trial, the primary intervention of this trial will be masked. Masking will reduce problems of compliance with the new intervention and reduce surveillance and ascertainment biases.

3.2.11. Study Specimens

We will obtain written informed consent from the parent(s) to collect stool samples weekly from birth to postnatal day 28 and a stool sample before hospital discharge. We will also obtain consent to collect a serum sample prior to hospital discharge to measure biomarkers of anabolism.

3.3. POTENTIAL RISKS AND BENEFITS TO SUBJECTS

This is non-exempt human subjects research.

Risk to Human Subjects

Human Subjects' Involvement, Characteristics, and Design. In this clinical trial, 150 study participants will be randomly assigned in a 1:1 allocation ratio to receive either a protein-enriched human milk diet (intervention group) or a usual human milk diet (control group) during the first 2 weeks after birth. Patients admitted to the UAB Neonatal Intensive Care Unit between July 2020 and December 2022 will be screened to determine eligibility for the trial. Our study population will reflect the epidemiology of prematurity in the state of Alabama, with approximately 51% being Black (African-American), 47% White (Non-Hispanic Caucasian), and the remaining 2% Hispanic/Other. This study population has been selected based on the frequency of postnatal growth problems observed in extremely preterm infants and the increased risk of adverse health outcomes in this vulnerable population.

Study Procedures, Materials, and Potential Risks. For study participants, data will be collected from electronic medical records. Data containing identifying information will be available only to the PI and research personnel directly involved with this study. Information about the study

will be shared without individual identifiers. Data will be collected from medical records in accordance with the study protocol. This will include demographic data and other nutrition/feeding data. This protocol will be reviewed by the Institutional Review Board of the University of Alabama at Birmingham.

Potential risks: The probability of risk for higher frequency of feeding interruptions in a patient that participates in this trial is not different than the probability of higher frequency of feeding interruptions in a patient that does not participate in the trial. Other theoretical risks of this study are related to clinical decompensation during assessment of infant body composition. They include increased risk of bradycardia or desaturations. Previous studies, including ours (unpublished data), have not reported an association between assessment of infant body composition and any of the above-mentioned risks. Similarly, studies of high-protein supplementation in preterm infants, primarily those fed formula did not report tolerance problems or increased risk of metabolic acidosis or high BUNs and it is unlikely that significantly adverse effects will be observed with excessive amounts of protein, particularly if the supplement is of high digestibility. Infants may experience some transient discomfort during the PeaPod® assessment which requires them to wear a tight-fitting cap but no other clothing or blanket. Therefore, there are no known risks associated with this trial except loss of confidentiality, as it involves data collection and imaging recording. This is one of the most common risks of participation in clinical research. Accordingly, our team has designed a strategy to protect participant confidentiality. All participants will be informed of study procedures and gauged for understanding of study tasks. In addition, study personnel will follow regulatory guidelines for obtaining informed consent and manage study data that includes personal information.

Alternative treatments and procedures: Participants in the intervention and control groups will have full access to all available standard of care clinical services at our neonatal unit, and parents are permitted to withdraw or refuse participation at any time. Serious adverse events will be reported to the DSMB and to the principal investigator.

Adequacy of Protection against Risks

Informed Consent and Assent. After a potential study participant is identified, a member of the study will see the parents and/or mother in the baby's room and explain the study. The risks and benefits will be discussed with the parents and time will be given to them to ask questions. It will be made known to them that no treatment will be withheld from their child if they participate in the study. The research team will attempt to obtain written consent after giving the parents a minimum of 24 hours to think about the study information and ask questions. Randomization will define study group assignment.

Protection against Risks. Our team will make every effort to protect all participants' confidential and private information in order to minimize possible study-associated risks. All findings related to any research will be available and provided to study participants in accordance with standard practices. We will also inform all participants that their participation is voluntary, and we will utilize study identification codes in place of personal identifiers on study materials. We will also employ storage and encryption techniques. All study personnel are required to renew Human

Subjects training biannually. No data will be accepted from or distributed to investigator or study staff if regulatory training is not current.

Vulnerable Subjects. This study population has been selected based on the frequency of postnatal growth problems observed in extremely preterm infants and the increased risk of adverse health outcomes in this vulnerable population. Because the probability of complications with protein-enriched human milk diets is not greater than the probability of complications with standard human milk diets, the category of children's risk level is 1 – research not involving greater than minimal risk. The risks of harm anticipated in the proposed research are not greater than those ordinarily encountered during the assessment of feeding tolerance.

Potential Benefits of the Proposed Research to Research Participants and Others

Potential benefits of this study include safety and efficacy data on early life protein-enriched human milk diets as a dietary intervention to prevent nutritional deficits, reduce postnatal growth failure, and increase fat-free mass accretion in preterm infants. Better quantitative and qualitative outcomes of growth may also be associated with improved long-term neurodevelopmental outcomes.

Reductions of postnatal growth failure and improved nutritional parameters could improve long-term neurodevelopmental outcomes of infants who survive to hospital discharge. There will be benefit to the medical community in providing additional information on infant body composition of preterm infants.

Importance of the Knowledge to be Gained

As the risk to individual participants is small and potential benefits are significant, the risk/benefit ratio is favorable. New knowledge on the effects of early diets on growth and the gut microbiome could measurably alter shift current practices and improve nutrition of preterm infants.

SECTION 4. ANALYTICAL PLAN

4.1. STATISTICAL ANALYSIS PLAN

All statistical analyses will use two-tail alpha to reject null hypotheses at 0.05, using R software. Continuous variables will be summarized as means ± standard deviations (SD) or as medians and interquartile ranges (IQRs). Categorical variable will be summarized as frequencies and proportions. Group differences will be evaluated using the T Test or Wilcoxon test for continuous variables and chi-square for categorical variables. The effect size of the primary outcome will be expressed as the mean difference with 95% confidence intervals (CIs). Risk ratios (RRs) with 95% CIs will be reported for categorical outcomes. All of the efficacy and safety outcomes of the trial will be analyzed with the intention-to-treat principle.

To analyze our longitudinal microbiome data, we will use either negative binomial models or zero-inflated models. Since there is no explicit formula to calculate power and detect taxa based differences on neither negative binomial nor zero-inflated models, we performed extensive simulation studies to assess the statistical power needed to identify significant taxa with the proposed trial. We employed the function sim in the R package NBZIMM (https://github.com/nyiuab/NBZIMM) to simulate longitudinal microbiome count data, and then used negative binomial mixed models to analyze the simulated counts. For the proposed longitudinal microbiome study design (i.e. with 75 infants in the intervention group and 75 infants in the control group that will have 3 to 6 stool samples collected over time), we will achieve ~80% power to detect ~ 2-fold effects. Since there is no explicit formula for analytically calculating power for detecting taxa based on neither negative binomial nor zero-inflated models, we performed extensive simulation studies to assess the statistical power to identify significant taxa with the proposed study design. We employed the function sim in the R package NBZIMM (https://github.com/nyiuab/NBZIMM) to simulate longitudinal microbiome count data, and then used negative binomial mixed models to analyze the simulated counts. To minimize possible bias and yield reasonable count values that are similar to real longitudinal microbiome data, we randomly generated the parameters (including the fixed fold-change effect, random effect, dispersion parameter, zero-inflation probability, etc.) in the model from reasonable ranges. For the proposed longitudinal microbiome study design (i.e. with 75 infants in the intervention group and 75 infants in the control group that will have 3 to 6 stool samples collected over time), we will achieve ~80% power to detect ~ 2-fold effects under a significance level of 5%.

For clinical and microbiome outcomes, adjusted analyses will be performed with the following covariates: volume intake in ml/kg, proportion of human milk intake, exposure to antibiotics after birth (i.e., number of days receiving antibiotics), race, sex, gestational age, and maternal use of antibiotics.

4.2. SAMPLE SIZE AND POWER ESTIMATES

We used our own institutional data from a previous enteral feeding trial to calculate the sample size for this trial. To detect a 0.5-difference in FFM-for-age Z-scores between groups with SD of 1, 0.05 level of significance, and 80% power for a T-test that compares means from

two independent samples, we estimated that a sample size of 126 patients will be necessary in this superiority trial. Anticipating that approximately 20% of study participants will be lost to follow-up for assessment of the primary outcome at 36 weeks PMA, we will add 12 patients to each group and increase the sample size to 150. We will include a total of 75 patients in each group (n=150).

4.3. AVAILABLE POPULATION

The estimated UAB available population based on inclusion/exclusion criteria is 150 per year.

4.4. PROJECTED RECRUITMENT TIME

Assuming a consent rate of 60%, this trial will require 2.5 years for patient recruitment and 1 year for completion of microbiome analyses. Therefore, the time to study completion is 3.5 years.

4.5. STUDY MONITORING PLAN

4.5.1. Reporting Adverse Events

Serious adverse events and suspected unexpected serious adverse reactions will be reported to the Data Safety and Monitoring Board (DSMB).

4.5.2. Data Monitoring Plan and Stopping Rules

Because not all neonatal units across the United States increase protein intake during the first 2 weeks after birth in extremely preterm infants, our DSMB will analyze all serious adverse events during the trial to determine whether they were result or consequence of participation in the trial. Unless modification or cessation of the protocol is recommended by the DSMB, the trial investigators will be unaware of the preliminary results described in these reports. Any provider or caregiver involved in the trial will be able to write to the DSMB to draw attention to any concern they may have about the possibility of harm arising from the intervention under investigation. The attending clinician may also withdraw the infant from the study if they consider this to be in the best interest of the infant's health and well-being.

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