Protocol Title:
Multisensory Early Oral Administration of Human Milk in Preterm Infants to Attenuate Early Life Toxic Stress on Epigenetic Modifications and Dysbiosis: Randomized Controlled Trial Pilot Study

Version Date: 05/07/2024

Table of Content

I. Research Team

Name and Credentials	Job Tittle	Affiliation	Role on the Research Team
Thao Griffith, PhD, RN	Assistant Professor	Loyola University Chicago	Principal Investigator
Linda Janusek, PhD, RN, FAAN	Professor Emerita	Loyola University Chicago	Consultant
Rosemary White-Traut, PhD, RN, FAAN*	Director	Children's Wisconsin, Nursing Research	Consultant
Sachin Amin, MD	NICU Director	Loyola University Medical Center	Site Principal Investigator
Cara Joyce, PhD	Director	Loyola University Chicago, Biostatistics Core, Clinical Research Office	Biostatistician
Talar Markossian, PhD, MPH	Associate Professor	Loyola University Chicago	Co-investigator
Julia Puchi, BSN, RN	Research Nurse	Loyola University Chicago	Research Nurse
Brianna Avery, BS	Research Assistant	Loyola University Chicago	Research Assistant
Jennifer Grunwaldt, MS, CCC-SLP	Speech Therapist	Loyola University Medical Center	Co-investigator
George Chlipala, PhD	Associate Director	University of Illinois at Chicago, Research Informatics Core	Consultant
Mark Maienschein-Cline, PhD	Director	University of Illinois at Chicago, Research Informatics Core	Consultant
Stefan Green, PhD	Director	Rush University Medical Center, Core Lab Services	Consultant
Barb O'Rourke, RN*	Registered Nurse	Blank Children's Hospital Synapse Care Solutions Reckitt-Mead Johnson	Consultant
Kristine Fuller, OTR/L, CNT, CLC*	Occupational Therapist	Caring Essentials Collaborative LLC Infant Feeding Strategies LLC	Consultant
*Rosemary White-Traut, Barb O'Rourke, and Kristy Fuller will not receive any PHI or other data from the study. Their role is to provide consultation on the protocol and logistics.			

II. Abstract

More than 60,000 infants are born between 22 to 32 weeks gestation age (very or extremely premature) annually in the US. Approximately 11% of them develop comorbidities, i.e., oral feeding difficulty, failure to thrive, delayed neurodevelopment, and maladapted parent-infant interaction. As part of the Neonatal Intensive Care Unit (NICU) hospitalization, very and extremely preterm infants inevitably endure early life toxic stress, e.g., parental separation, painful procedures, sleep fragmentation, harmful hospital organisms, and antibiotic therapy, without adequate protective buffers. Early life toxic stress results in adverse epigenetic modifications of glucocorticoid-related genes (DNA methylation of NR3C1 and HSD11B2) and dysbiosis (altered gut microbiome), both impairing neurodevelopment. These adversities further exacerbate the risk of comorbidities and inappropriate brain development during sensitive periods of neuroplasticity. Evidence suggests adverse epigenetic modifications and dysbiosis may set a life-long trajectory of risk for chronic health conditions, i.e., mental health disorders, cardiometabolic and immunologic diseases. Hence, it is a clinical and scientific priority to test an early NICU intervention to attenuate stress-related adverse epigenetic modifications and dysbiosis. Prior research has paved the way for a potential early NICU intervention as follows. Human milk influences the structure and relative abundance of healthy gut bacteria, i.e., short-chain fatty acid (SCFA)- and lactate-producing bacteria (e.g., Bifidobacterium and Lactobacillus), and neurodevelopment. Maternal nurturing, e.g., licking and grooming (in rodents), and breastfeeding and touch (in humans), promotes neurodevelopment, reduces stress, and reverses stress-related epigenetic modifications. Building on what is known, the multisensory early oral administration of human milk (M-MILK) intervention by nurses or parents is designed to provide an enjoyable and nurturing experience for infants, through a safe and consistent infant-guided provision of human milk droplets, given orally as early as 22 weeks postmenstrual age. M-MILK is implemented after every hands-on care and during the beginning of a full gavage feeding. The objective of our planned NIH R01 randomized controlled trial is to examine the efficacy of M-MILK from birth to 2 months corrected age, and the mechanisms whereby this may occur. We propose the M-MILK pilot study (N = 20): a 2-group, 10 per group, parallel design, and longitudinal randomized controlled trial in preterm infants who are born between 22 to 33 weeks gestational age. M-MILK will begin on day 3 of life. We will measure outcomes using validated methodologies: maternal postpartum depressive symptoms via the Edinburgh Postnatal Depression Scale, parent stress via the Parental Stressor Scale: NICU, parent discharge readiness via the Screening and Multi-disciplinary Risk Assessment Scale & Parent Activation Scale, neurodevelopment via the Neurobehavioral Assessment of the Preterm Infants and Ages & Stages Questionnaire-3, oral feeding skills via the Early Feeding Skills Assessment and NeoEAT, parent-infant interaction during feeding via the Nursing Child Assessment Satellite Training–Feeding Scale and Postpartum Bonding Questionnaire, DNA methylation of NR3C1 and HSD11B2 via buccal samples, and gut microbiome via fecal samples. Outcomes will be measured at baseline, 34 weeks postmenstrual age, discharge, and 2 months corrected age. The aims of this pilot study are to (1) determine the feasibility and acceptability of the M-MILK intervention by nurses and parents, (2) determine the feasibility of recruitment and retention, (3) obtain variability data for sample size estimation, and (4) evaluate the charges and costs of the NICU hospitalization and 2 months CA care between the M-MILK and control groups. M-MILK will be considered feasible if M-MILK is implemented by nurses or parents after every hands-on care and during the beginning of a full gavage feeding at least 90% of the time. The M-MILK acceptability by nurses and parents will be determined via M-MILK Acceptability Questionnaire. Recruitment and retention will be considered feasible if at least 50% of infants approached for participation enrolled, 90% of infants completed all inpatient data collection, and at least 50% of infants completed post-discharge follow-ups. This pilot study will inform our R01 application regarding the feasibility and acceptability of M-MILK and sample size estimation.

III. Background

More than 60,000 infants are born between 22 to 32 weeks gestation age (very or extremely premature) annually in the US.[1] Approximately 11% of them develop comorbidities, i.e., oral feeding difficulty, failure to thrive, delayed neurodevelopment, and maladapted parent-infant interaction.[2-7] As part of the Neonatal Intensive Care Unit (NICU) hospitalization, very and extremely preterm infants inevitably endure early life toxic stress, e.g., parental separation, painful procedures, sleep fragmentation, harmful hospital organisms, and antibiotic therapy.[7-9] We define early life toxic stress in the NICU as excessive and prolonged stressful stimuli without buffer of a consistent, supportive, and protective parental relationship.[7,10] Early life toxic stress results in adverse epigenetic modifications of glucocorticoid-related genes (DNA methylation of *NR3C1* and *HSD11B2*)[11-13] and dysbiosis (altered gut microbiome),[14-17] both impairing neurodevelopment.[11,13-15,18] These adversities further exacerbate the risk of comorbidities and inappropriate brain development during sensitive periods of neuroplasticity.[7,19] Evidence suggests adverse epigenetic modifications and dysbiosis may set a life-long trajectory of risk for chronic health conditions, i.e., mental health disorders, cardiometabolic and immunologic diseases.[8,19-21] Hence, it is a clinical and scientific priority to test an early NICU intervention to attenuate stress-related adverse epigenetic modifications and dysbiosis. Prior research has paved the way for a potential early NICU intervention as follows. Human milk influences the structure and relative abundance of the healthy gut bacteria, i.e., short-chain fatty acid (SCFA)- and lactate-producing bacteria (e.g., *Bifidobacterium* and *Lactobacillus*), and neurodevelopment.[12,22,23] Maternal nurturing, e.g., licking and grooming (in rodents) and breastfeeding and touch (in humans), promotes neurodevelopment, reduces stress, and reverses stress-related epigenetic modifications.[13,21,24-26] Building on what is known, the multisensory early oral administration of human milk (M-MILK) intervention by nurses or parents is designed to provide an enjoyable and nurturing experience for infants, through a safe and consistent infant-guided provision of human milk droplets, given orally as early as 22 weeks postmenstrual age.[27,28] M-MILK is implemented after every hands-on care and during the beginning of a full gavage feeding.[28] The objective of our planned NIH R01 randomized controlled trial is to examine the efficacy of M-MILK from birth to 2 months corrected age, and the mechanisms whereby this may occur.

Initial evidence from prior studies is promising, however, the study designs lack rigor and comprehensiveness. In these studies, early administration of mother's own milk or donor's milk shortly after birth supported oral mechanical stimulation and subsequent maturation of suckling and swallowing skills.[28,29] It is evidenced by less episodes of feeding intolerance and shorter duration to full oral feeding.[28,29] Researchers also found that the intervention offered immunoprotection as evidenced by lower pathogenic microbacterial colonization of the gastrointestinal tract and incidence of ventilator-associated pneumonia.[29] The intervention reduced duration of oxygen support, length of stay, and cost of care.[27-30] However, the potential efficacy of M-MILK to attenuate the effects of early life toxic stress on epigenetic modifications and gut microbiome is unknown. This gap leads to a missed opportunity for individualized neonatal care and early parental involvement in the NICU which could protect very and extremely preterm infants against early life toxic stress. There is an urgent need to address this knowledge gap and determine the efficacy of M-MILK.

To inform our planned R01 application, we propose the M-MILK pilot study (N = 12): a 2-group, 6 per group, parallel design, and longitudinal randomized controlled trial in infants who are born between 22 to 33 weeks gestational age. We will compare the outcomes between the M-MILK and attention control groups. M-MILK will begin on day 3 of life. We will measure outcomes using validated methodologies, e.g., maternal postpartum depressive symptoms via the Edinburgh Postnatal Depression Scale, parent stress via the Parental Stressor Scale: NICU, parent discharge readiness via the Screening and Multi-disciplinary Risk Assessment Scale & Parent Activation Scale, neurodevelopment via the Neurobehavioral Assessment of the Preterm Infants and Ages & Stages Questionnaire-3, oral feeding skills via the Early Feeding Skills Assessment and NeoEAT, parent-infant interaction during feeding via the Nursing Child Assessment Satellite Training–Feeding Scale and Postpartum Bonding Questionnaire, DNA methylation of *NR3C1* and *HSD11B2* via buccal samples, and gut microbiome via fecal samples. Outcomes will be measured at baseline, 34 weeks postmenstrual age, discharge, and 2 months corrected age. The aims of this pilot study are to (1) determine the feasibility and acceptability of the M-MILK intervention by nurses and parents, (2) determine the feasibility of recruitment and

retention, (3) obtain variability data for sample size estimation, and (4) evaluate the charges and costs of the NICU hospitalization and 2 months CA care between the M-MILK and control groups.

This innovative pilot study will be the first to examine M-MILK on a cumulative scale of clinical and molecular outcomes of infants and parents. The findings will yield novel insights regarding an early NICU intervention that is clinically safe, effective, and scalable. By promoting epigenetic protection and healthy gut microbiome, M-MILK has significant potential to improve future health outcomes and reduce the medical and financial burdens across the lifespan for very and extremely preterm infants.

IV. Study Aims

The aims of this pilot study are to (1) determine the feasibility and acceptability of the M-MILK intervention by nurses and parents, (2) determine the feasibility of recruitment and retention, (3) obtain variability data for sample size estimation, and (4) evaluate the charges and costs of the NICU hospitalization and 2 months CA care between the M-MILK and control groups. M-MILK will be considered feasible if M-MILK is implemented by nurses or parents after every hands-on care and during the beginning of a full gavage feeding at least 90% of the time. The M-MILK acceptability by nurses and parents will be determined via M-MILK Acceptability Questionnaire. Recruitment and retention will be considered feasible if at least 50% of infants approached for participation enrolled, 90% of infants completed all inpatient data collection, and at least 50% of infants completed post-discharge follow-ups.

V. Administrative Organization

Participants will be enrolled in the Neonatal Intensive Care Unit (NICU) at Loyola University Medical Center (LUMC) (Maywood, IL). The inpatient components of the study protocol will be conducted in the NICU at LUMC. The post-discharge follow-up components of the study protocol will be conducted via phone/zoom interview. Data storage and management will be carried out via REDCap (version 10.4.0, Vanderbilt University) and a secured research network drive, hosted by the Loyola University Chicago (Maywood, IL).

Upon sample collection, buccal swab and fecal samples will be labeled only with study ID numbers and collection date, and stored in a locked cabinet in a locked laboratory at Loyola University Chicago (LUC) Center for Translational Research and Education (CTRE). All samples will be stored at room temperature. Quarterly, the buccal swab samples and fecal samples will be securely transported in a biospecimen transfer container (Saf T Pak, Austell, GA) via car to Rush University Medical Center Genomics and Microbiome Core Facility (Rush) (Chicago, IL) for processing and analyses. A Service Agreement between LUC and Rush will be submitted through LUC Research Portal.

VI. Study Design

a. Design

The M-MILK pilot study (N = 12) is a 2-group, 6 per group, parallel design, and longitudinal randomized controlled trial. We will recruit preterm infants who are born between 22 to 33 weeks gestational age. We will compare the outcomes between the M-MILK and attention control groups. Infants will be enrolled into the study as soon as possible after they are admitted to the NICU. If infants are in the M-MILK group, M-MILK will begin on day 3 of life after every hands-on care and during the beginning of a full gavage feeding. We will measure outcomes using validated methodologies at baseline (enrollment), 34 weeks PMA, discharge, and 2 months CA.

b. Setting

The M-MILK pilot study will be conducted in a level III NICU at LUMC (Maywood, IL). The hospital provides care to families with diverse racial, ethnic, and socioeconomic backgrounds. The nursing staff are predominantly registered nurses and advanced nurse practitioners, and the physicians are board certified

neonatologists. Infants are fed using mother's own milk, donor milk, and/or formula. However, we will only include infants who receive mother's own milk or donor's milk.

c. Sample

We will enroll 12 preterm infants (6 per group). Anticipated a 40% attrition rate, we expect the final sample size to be 12 preterm infants. Estimates of average values and variability of continuous measures are acceptable at this sample size using the "rule of 12" as recommended.[31]

VII. Study Procedures

a. Subject Selection Procedures

i. Inclusion/Exclusion Criteria

Inclusion criteria

- Born between 22 to 33 weeks gestational age
- Mother is pumping and infant is receiving mother's own milk and/or donor's milk to be in the intervention group.

Exclusion criteria

- Congenital abnormalities in the digestive, e.g., clef lip and palate, omphalocele
- Necrotizing enterocolitis
- Congenital heart disease that requires surgery

ii. Screening procedures

After infants are admitted to the NICU, we will review their electronic medical records to screen for eligible infants according to the inclusion and exclusion criteria. Once an infant is enrolled into the study, the screening period continues until NICU discharge. The infant will be classified as screen failed and excluded if he/she develops any of the exclusion criteria.

iii. Recruitment Procedures

Parents of eligible infants will be approached during their post-partum hospitalization or in the NICU at LUMC. A research team member will provide details regarding the study purpose, procedures, and answer questions. Parents will be provided with a study brochure. If parents request additional time to make their decision, we will request a phone number and ask permission to follow-up. If parents agree to have their infant participate, a written informed consent and authorization to use and disclose the PHI will be obtained. Parents will receive a copy of their signed informed consent and authorization to use and disclose the PHI. A copy of the informed consent form will be uploaded to the infant's electronic medical record. The original signed consent will be stored in a locked cabinet at LUC CTRE (Maywood, IL). Infant's participation in the study will end after the phone/Zoom interview at 2 months CA. We will provide a $50 incentive e-gift card at NICU discharge and a $50 incentive e-gift card at the completion of the post-discharge follow-up, a total of $100 e-gift card. Premature infants, by virtue of their age, are unable to give consent or assent, and therefore, will not give assent or participate in the informed consent process.

iv. Randomization Procedures

Randomization is controlled by using two computer-generated lists of random numbers that are corresponded to each group: Group 1 (M-MILK) and Group 2 (attention control), balanced so that 1/2 of the infants are in each group. The lists are retained. The random numbers will be printed in order on small cards stated as Group 1 or Group 2. The cards will be placed in envelopes and placed in order in a box. Prior to approaching parents for recruitment, we will open the first envelope in the box to obtain the group assignment. We will request parents to not disclose their group assignment with other parents in the NICU. The research team

members, staff nurses, and parents cannot be blinded to the treatment condition. The research team members need to know which intervention to provide. Staff nurses provide direct care to infants. The two groups are described in the consent form as required by LUC Institutional Review Board.

v. Study Intervention

1. MMILK Intervention

The M-MILK group will have access to the educational materials regarding preterm infant care such as Your Guide to Neonatal Intensive Care Unit, Breastfeeding in the NICU, and M-MILK Toolkit video. We will provide parents with two scent hearts to wear and bring back to place near the infant's face. Parents will perform routine care such as taking temperature, bathing, changing diapers, reading, feeding, holding hands, as well as M-MILK when appropriate. Parents will receive an automatic daily text in the morning with a link to report their daily activities with infants when they visit the NICU.

The M-MILK Intervention Protocol is a modified and improved version of previous protocols. This M-MILK protocol is carefully designed by a team of neonatal experts, with a strong focus on developmentally appropriate, trauma informed care, and family friendly language. M-MILK is implemented at day 3 of life even if the infant requires respiratory support and/or mechanical ventilation. M-MILK is implemented after every hands-on care and during the beginning of a full gavage feeding. Infants receive M-MILK as droplets via a 1-ml syringe. In our NICU, oral feeding attempts are initiated at approximately 33 to 34 weeks PMA and based on the infant's pre-feeding cues. M-MILK is not offered with oral feeding attempts since the feeding itself is considered a positive experience. We will use either mother's own milk and or donor's milk depending on availability. Infants may receive up to 1 mL of milk each time based on their cues and responses. The 1 mL volume intake is included as part of their oral caloric intake. M-MILK is provided by the infant's nurse or parents. Parents are encouraged and supported to provide M-MILK when appropriate with the nurse's supervision.

Intervention Training and Fidelity. Delivery of M-MILK by nurses or parents introduces potential threats to intervention fidelity. We will take the following strategies for training and improving intervention fidelity. 1) In-person 1-hour M-MILK training sessions for all nurses, led by the PI and the M-MILK Expert Consultant, held in groups quarterly and individually as needed. The M-MILK training sessions will cover the M-MILK Toolkit. An acceptable attendance rate during the first quarter is 90%. 2) M-MILK Toolkit is available for nurses to view on the M-MILK study website and LUMC HealthStream platform for reference and continue education. 3) Nurses will document infant's responses to M-MILK; and research team will review M-MILK documentation by nurses daily to ensure accuracy and completion. 4) In-person 30-minute M-MILK training sessions for parents in the M-MILK group, within 1 week of enrollment, provided by a Research Nurse. The M-MILK training sessions will cover the M-MILK Toolkit. The M-MILK training sessions for parents are followed by in-person observation and hands-on return demonstration, and questions/answers with a Research Nurse. An acceptable attendance rate for the parent M-MILK training sessions is 90%. 6) M-MILK Toolkit is available on the M-MILK study website for reference and continuing education. 7) Access to the M-MILK content on the website is restricted to nurses and parents in the M-MILK group, thus there will be 2 different access codes for each group. 8) We will request parents not to share the M-MILK content with other parents in the NICU. 9) Parents will document the M-MILK activities and infant's responses via the daily activity link; acceptable completion rate for parent documentation is 85%. 10) Research Nurse will conduct an in-person audit of 10% of the M-MILK administrations by nurses or parents quarterly using M-MILK Intervention Fidelity Checklist; acceptable reliability rate is 90%. 11) The Research Nurse who is responsible for studying main outcome evaluations will be blinded to the infant's group assignment.

2. Attention Control Group

For infants in the attention control group, standard of care will be provided, including hands-on care by their nurses, diapering, swaddling, feeding, etc. After assessment and initial cares are completed, infants are tucked and nested with boundaries to support comfort, security, and flexion. The infants' hands are placed by their faces to encourage self-soothing, mouthing, and sucking. We will provide parents with two scent hearts to wear

and bring back to place near the infant's face. Parents perform routine care such as taking temperature, bathing, changing diapers, reading, feeding, and holding hands. Parents will have access to educational materials regarding preterm infant care such as Your Guide to Neonatal Intensive Care Unit and Breastfeeding in the NICU. Parents will receive an automatic daily text in the morning with a link to report their daily activities with infants when they visit the NICU. Parents in the attention control group will not have access to the M-MILK content.

vi. Study Assessments and Activities

1. Measures

Table 1. Concepts, Measures, and Operational Definitions

Concepts	Operational Definitions	Data Source & Time
Parent characteristics	Parent characteristics will include both mother and father as applicable. We will collect race/ethnicity, education, age at delivery, household income, number of children, number of children under 24 months, and social determinants of health (i.e., physical activity, financial resource strain, housing stability, transportation needs, food insecurity, stress, social connections, intimate partner violence, and alcohol use)	T0 EPIC & self-report
Maternal postpartum depressive symptoms	Maternal postpartum depressive symptoms are assessed by the Edinburgh Postnatal Depression Scale (EPDS).[32] The EPDS consists of 10 short statements. A mother checks off one of four possible answers that is closest to how she has felt during the past week. Responses are scored 0, 1, 2 and 3 based on the seriousness of the symptom. Items 3, 5 to 10 are reverse scored (i.e., 3, 2, 1, and 0). The total score is calculated by adding together the scores for each of the 10 items. Mothers scoring above 12 or 13 are likely to be suffering from depression and should seek medical attention. The pooled sensitivity and specificity of the EPDS were 0.81 and 0.87, respectively, with summary receiver operating characteristic curve of 0.90.[33]	T0, T3, T4 EPDS
Parent stress	We will also measure parent stress associated with their infant's NICU hospitalization via the Parental Stressor Scale: NICU (PSS: NICU).[34] The PSS:NICU measures parental stress after preterm birth and admission of their neonate to NICU. It is a self-report questionnaire, which can be rated on a five-point Likert-Scale: 1 = not at all stressful: the experience did not cause you to feel upset, tense, or anxious, 2 = a little stressful, 3 = moderately stressful, 4 = very stressful, and 5 = extremely stressful. Items describing situations that have not been experienced by the parents can also be answered with "not applicable." The PSS: NICU has good concurrent and predictive validity and is internally consistent with Cronbach alpha ranging from 0.73 to 0.94.[35]	T3 PSS: NICU & Self-report
Parent discharge readiness	Parent discharge readiness will be evaluated by the Screening and Multi-disciplinary Risk Assessment Scale & Parent Activation Scale (PDR). The scale has 44 items and includes five sections: self-efficacy, social support, outcomes expectations and intent, knowledge, and perception of risk. The content validity of the scale is supported.[36]	T3 PDR & Self-report
Postpartum Bonding Questionnaire	Parent-infant bonding will be evaluated by the Postpartum Bonding Questionnaire (PBQ). The PBQ comprises four subscales, i.e., general bonding disorders (12 items; 1, 2, 6, 7, 8, 9, 10, 12, 13, 15, 16, 17), severe mother-infant relationship disorders (7 items; 3, 4, 5, 11, 14, 21, 23), infant-focused anxiety (4 items; 19, 20, 22, 25), and risk of abuse (2 items; 18, 24). The total cumulative score, ranging from 0 to 125, is also used to screen for general bonding disorders (cut-off score ≥26), and severe bonding disturbances (cut-off score ≥40). The items are scored on a 6-point Likert scale (0-Always to 5-Never). Parents scoring ≥ 26, (indicating risk of general bonding disorders) or ≥ 40 (indicating risk of severe bonding disturbances) should seek medical attention. Parents scoring "rarely", "sometimes", "quite often", "very often", or "always" for item 18 "I have done harmful things to my baby" or item 24 "I feel like hurting my baby" indicates risk of child abuse. In this case, it is our responsibility to report it to the state's child protective agency. If the infant is in immediate danger, the research staff will be instructed to inform the parents of their immediate concerns for the infant's safety, report the incident using a cellular phone, and call 911 until appropriate officials arrive. The PI will be informed of the family's condition and always be available by phone or in person for consultation and instruction. As part of the training of our research team, the team will review content related to reporting of child neglect and abuse.	T1, T3, T4 PBQ & Self-report
Pumping and breastfeeding	Mothers will report the following: pumping initiation date, pumping cessation date, pumping at discharge (yes/no), pumping at 2 months CA (yes/no), breastfeeding initiation date, breastfeeding cessation date, breastfeeding at discharge (yes/no), & breastfeeding at 2 months CA (yes/no).	Ongoing Self-report

Family daily NICU activities	Parents will report the following: (1) How many hours were you able to visit your baby today? (2) What activities did you do with your baby while you were visiting? The answer choices for question (2) for parents in the M-MILK group are: M-MILK, skin-to-skin, feeding, taking temperature, bathing, changing diaper, reading, holding hands.	Ongoing Self-report
Infant characteristics	race/ethnicity, & sex, GA at birth, birthweight, 5-minute Apgar score, small for gestational age (yes/no), intra-uterine growth restriction (yes/no), PROM (yes/no), medical diagnoses & therapies, daily weight gain, PMA at discharge, duration of time from initiation of oral feeding to discharge, & duration of NICU hospitalization; daily oral feeding intake, daily type of milk (mother's own milk, donor's milk, or formula), duration of tube feeding, duration of time from initiation of oral feeding to full oral feeding, & PMA at initiation & full oral feeding.	Ongoing EPIC
Infant medical severity	Infant medical severity will be measured via the Neonatal Medical Index Classification (NMI), range 1-5, 1 as absence of serious medical problems & 5 as the most serious complications.[37]	T3 EPIC
Early life stress	Early life stress will be measured daily by the modified Neonatal Infant Stressor Scale (NISS)[38] using data from the EPIC during the entire NICU hospitalization. The Neonatal Infant Stressor Scale (NISS):[38] summary scores, 47 acute interventions & 23 chronic conditions, weighted from 2-5, 2 as 'a little stressful' & 5 as 'extremely stressful'.[39] Predictive & construct validity were established, i.e., correlations between higher stress scores & brain structural changes,[40] & poorer neurobehaviors.[38,41]	Ongoing EPIC
Neurodevelopment	Neurodevelopment will be evaluated via the Neurobehavioral Assessment of the Preterm Infants (NAPI) at discharge.[42-45] The PI will train a Research Nurse to criterion of ≥ 90% agreement. A Research Nurse (who is blinded to the infant's group assignment) will conduct, rate and video record the NAPI assessment. A secondary trained evaluator will code 20% of the videos to assure continued reliability. The Neurobehavioral Assessment of the Preterm Infants (NAPI, 73 items, 32-37 weeks PMA): summary scores, normed referenced means, standard deviation, 7 clusters: scarf sign, motor development & vigor, popliteal angle, alertness and orientation, irritability, quality of cry, & percent sleep ratings.[42-45] Construct validity, face validity, & sensitivity of the NAPI were described using an index of medical complications & general movements.[43,44,46] Inter-rater reliability coefficient was 0.67 to 0.97.[47]	T3 NAPI
	Neurodevelopment will also be evaluated via the Ages and Stage Questionnaire (ASQ-3), completed by parents at 2 months CA.[48] The ASQ-3 is a developmental screening tool that pinpoints developmental progress in children between the ages of one month to years. It is a validated and standardized screening tool based on parental report that reliably assesses five key developmental domains: communication, fine and gross motor, problem solving, and personal-social skills. The ASQ-3) has strong concurrent validity (r = 0.85), 2-week test-retest reliability (r = 0.75-0.82), interobserver reliability (r = 0.43-0.69), and internal consistency (α = 0.51-0.87).[48] The ASQ-3 provides cutoff scores to indicate possible delay as follows: communication, gross motor, fine motor, problem solving, and personal-social. The AAP reports the ASQ-3 sensitivity range to be 0.70 to 0.90 and specificity to be 0.76 to 0.91.[49] The five scored domains include communication, gross motor, fine motor, problem-solving, and personal–social. Scores of 10, 5, and 0 are applied, respectively, to caregiver responses of "yes," "sometimes," and "not yet" to 30 items. The scores are tabulated for each domain and are further categorized into a color-coded chart based on score interpretation. "Above cutoff" is considered typical development and is defined as any score in the white area (higher than 1 SD below the mean); scores within the gray area indicate the "monitoring zone" in which the child should be observed and another screening may be desirable in a few months (1–2 SD below the mean); and scores within the black area are "below cutoff" and indicate the child may be at risk for developmental delays and should be referred for further assessment (2 SD below mean).[50]	T4 ASQ-3
Oral feeding skill development	Oral feeding skill development will be evaluated using the Early Feeding Skill Assessment (EFS) at oral feeding initiation and discharge.[51] The PI will train a Research Nurse to criterion of ≥ 90% agreement. A Research Nurse (who is blinded to the infant's group assignment) will video record the feeding and rate the EFS. A secondary trained evaluator will code 20% of the videos to assure continued reliability. EFS has 22 items (32-50 weeks PMA), summary scores, 5 subscales: respiratory regulation, oral-motor functioning, swallowing coordination, engagement, & physiologic stability.[51] Interrater reliability & construct validity have been established, i.e., Cronbach α = 0.81, correlations among EFS scores, GA & PMA.	T2, T3 EFS
	Oral feeding skill development will also be evaluated using the NeoEAT at 2-month CA.[52,53] There are 2 versions of the NeoEAT: bottle-feeding and breast-feeding and will be offered accordingly. The NeoEAT will be completed via during the home visit by a caregiver who is familiar with the infant's eating. Neonatal Eating Assessment Tool– Bottle-feeding (NeoEAT–Bottle-feeding, 64 items, less than 7 months old, parent-report): summary scores, 5 subscales: regulation, energy & physiologic stability, gastrointestinal tract function, sensory responsiveness, & compelling symptoms of problematic feeding.[53] Internal consistency reliability (α = 0.92) and test-retest reliability (r = 0.90; P < .001) were excellent. Construct	T4 NeoEAT

	validity was established with the I-GERQ-R (r = 0.74; P < .001) and IGSQ (r = 0.64; P < .001). Healthy infants scored lower on the NeoEAT – Bottle-feeding than infants with feeding problems (P < .001), supporting known-groups validity. Neonatal Eating Assessment Tool – Breast-feeding (NeoEAT – Breast-feeding, 62 items, less than 7 months old, parent-report): summary scores, 7 subscales: regulation, energy & physiologic stability, oral-pharyngo-esophageal function, gastroesophageal function, gastrointestinal function, feeding efficiency & sensory responsiveness, & compelling symptoms of problematic feeding.[52] Internal consistency reliability (Cronbach's α = .92) was excellent. Test-retest reliability (r = 0.91) was high. Concurrent validity was established with the Infant Gastroesophageal Reflux Questionnaire (r = 0.69) and Infant Gastrointestinal Symptoms Questionnaire (r = 0.62). The NeoEAT-Breastfeeding total score and all subscale scores were higher in infants with feeding problems than in typically feeding infants (p < .001), supporting known-groups validity.	
Parent-Infant Interaction during Feeding	The Nursing Child Assessment Satellite Training–Feeding Scale (NCAST-Feeding) will be used to assess behaviors between infants and mothers at oral feeding initiation, NICU discharge, and 2 months CA. The PI will train a Research Nurse to criterion of ≥ 90% agreement. A Research Nurse will video record the feeding interactions. A Research Nurse (who is blinded to the infant's group assignment) will code the feeding interactions from the videos. A secondary trained evaluator will code 20% of the videos to assure continued reliability. The Feeding Scale (76 items) consists of six subscales: (a) maternal-sensitivity to cues; (b) maternal response to distress; (c) maternal social-emotional growth-fostering; (d) maternal cognitive growth fostering; (e) infant clarity of cues; and (f) infant responsiveness to caregiver.[54-59] Parent infant interaction will be video recorded. The NCAST has been widely used in parenting research and has well established reliability and validity for term and preterm infants. For preterm infants, the child subscales are significantly correlated to later IQ. Internal consistency is high for the total score (α = 0.86), the mother total score (α = 0.83) and for the infant total score (α =.73). Concurrent validity is documented using the Teaching Scale and the Home Observation of the Environment (HOME). Construct validity is evidenced by the subscales' ability to identify clinical problems. For example, if the child has been premature the child's score is low, while if the parent has a problem such as low education the mother's score is low.	T2, T3 NCAST-Feeding
DNA methylation of *NR3C1* & *HSD11B2*	We will evaluate DNA methylation in buccal cell DNA collected at baseline, 34 weeks PMA, NICU discharge, and 2 months CA, as an index of early life toxic stress. A trained research team member will be collecting, transporting, and storing the samples. We will collect two buccal swab samples from each infant at each visit using Isohelix DNA Buccal Swabs (Boca Scientific, Westwood, MA) before feeding to avoid contamination. The buccal swab samples will immediately be placed in a storage tube with a DNA stablizer Dri-Capsule (Boca Scientific, Westwood, MA). The samples collected will then be transported to a laboratory at the Loyola University Chicago Center for Translational Research and Education (Maywood, IL). All samples will be stored at room temperature. Quarterly, the buccal swab samples will be securely transported in a biospecimen transfer container (Saf T Pak, Austell, GA) via car to Rush University Medical Center Genomics and Microbiome Core Facility (Chicago, IL) for processing and analyses. DNA will be extracted from buccal swab samples using the Maxwell® RSC Buccal Swab DNA Kit (Promega, Maddison, WI) implemented on an automated Promega RSC device. The DNAm status for both the *NR3C1* exon 1F and *HSD11B2* promoter regions will be assessed using quantitative bisulfite amplicon sequencing using an Illumina MiSeq sequencer. Preparation of the bisulfite-converted DNA for sequencing will be accomplished with primers for *NR3C1* and for *HSD11B2*, as described,[11] employing a two-stage polymerase chain reaction protocol.[60] Using the Illumina MiSeq sequencer, we will be able to sequence the entire targeted region, including the 13 *NR3C1* CpG sites in the 1F region and the 4 *HSD11B2* CpG sites. These sites are associated with neuroendocrine system regulation, including the HPA axis, and cortisol reactivity in rodent and human studies.[21,61-63] Bisulfite conversion controls will be included with each sequencing run and will also be assessed for non-CpG 'C's. Samples with conversion rates below 93% will be re-processed. Libraries will be prepared in technical triplicate from each bisulfite converted DNA template, and if the replicates differ by > 10%, the sample analysis will be repeated. Unmethylated CpG sites will be converted to UpG (TpG upon PCR amplification) by bisulfite treatment. The percent methylation at any nucleotide coordinate will be determined by the ratio of C residues (derived from 5-methylcytosine) to C+T residues (derived from 5-methylcytosine plus cytosine, respectively) at any position within the *NR3C1* and *HSD11B2* genomic DNA sequence. In addition, the methylation status of each read will be assessed individually by counting the number of methylated CpG sites observed within that read and summed to assess average methylation status in each amplicon. The percent DNAm at the *NR3C1* and *HSD11B2* promoters and at each CpG site will be quantified using CLC genomics workbench (Qiagen) software package. DNAm data will be analyzed as a	T0, T1, T3, T4 Buccal samples

	continuous measure based on total percent of methylation for each *NR3C1* and *HSD11B2* promoter region as well as for each individual CpG site.	
Gut Microbiome	Infant fecal samples will be collected by a bedside nurse in the NICU at baseline, 34 weeks PMA, NICU discharge. For the 2 months CA sample, parents will receive a fecal collection kit with written instructions to collect and send samples to our lab via USPS postal services. Fecal samples from each infant will be collected using sterile, disposable spatulas during diaper changes and then placed into a sterile specimen container with microbial DNA stabilizer solution for 60 days at room temperature. The samples collected will be transported to a laboratory at the Loyola University Chicago Center for Translational Research and Education (Maywood, IL). All samples will be stored at room temperature. Monthly, the buccal swab samples will be securely transported in a biospecimen transfer container (Saf T Pak, Austell, GA) via car to Rush University Medical Center Genomics and Microbiome Core Facility (Chicago, IL) for processing and analyses. Genomic DNA will be extracted from fecal material using a Maxwell RSC48 autM-MILKted extraction device (Promega) and employing a Maxwell RSC Fecal Microbiome DNA kit, as described previously.[64] Samples will be subject to physical shearing (i.e., bead-beating) using a TissueLyser II device (Qiagen) prior to extraction. Genomic DNA will be prepared for sequencing using a two-stage amplicon sequencing workflow[65] and employing primers 515F-modified and 806r-modified.[66] Sequencing will be performed a MiniSeq sequencer (Illumina)[64] with paired-end 2x154 base sequencing. Basic processing of sequence data, including merging of forward and reverse reads, quality and primer trimming, chimera removal, and selection and annotation of amplicon sequence variants (ASVs) will be performed.[64] Alpha diversity indices (*e.g.*, Shannon index) will be calculated on rarefied datasets[67] in R using the vegan library package.[68] Pairwise comparisons of alpha diversity indices will be performed using the non-parametric Mann–Whitney test. Beta diversity analyses will be performed on pairwise Bray–Curtis indices calculated in R using the metaMDSdist function in the vegan library v2.5-6. The dissimilarity indices will be modelled and tested for significance with the sample covariates using PERMANOVA.[69] Differential abundance analyses of individual taxa between groups will be performed using the software package DESeq2, generating an FDR *q*-value.[70] DESeq2 has been shown to be appropriate for differential abundance comparisons in studies with small sample size groups (< 20) or unbalanced design.[71] To identify taxa that most strongly explain between-group differences, a Linear discriminant analysis Effect Size (LEfSe) analysis will performed.[71]	T0, T1, T3, T4 Fecal samples
Charges and costs of care	Healthcare use and associated charges and costs of the NICU hospitalization and 2 months CA care will be extracted from LUMC's electronic health and billing records. For infants receiving care outside LUMC 2 months CA, we will ask the parents about the extent and types of healthcare received outside LUMC. We will evaluate healthcare use, costs, and charges between the M-MILK and control groups. We will also describe the extent and types of healthcare use 2 months CA for infants receiving care outside LUMC at discharge. In the R01 trial, we will use activity-based costing approach to calculate the time and resources costs of delivering the M-MILK intervention by nurses and parents (which may not be fully reflected in the health system billing records), and query NICU hospitalization and 2 months CA healthcare use, charges, and costs to calculate total benefits and costs of M-MILK Intervention vs. control group.	T3 & T4
M-MILK Acceptability Questionnaire	The M-MILK Acceptability Questionnaire will be completed by parents at discharge. The nurses will complete the M-MILK Acceptability Questionnaire at the end of the entire study.	T3 M-MILK Acceptability Questionnaire

Note. EPIC = electronic medical record; GA = gestational age; PMA = postmenstrual age; CA = corrected age; Ongoing = throughout hospitalization from enrollment to discharge; T0 = baseline; T1 = 34 weeks PMA; T2 = oral feeding initiation; T3 = discharge; T4 = 2 months CA

ii. Procedures

Figure 2. After informed consent is obtained, infants will be randomized into: Group 1 (M-MILK) and Group 2 (attention control). All data collection will be conducted in the same manner and at the planned time points for both groups. For all infants, we will collect infant characteristics, NISS, and family NICU activities daily ongoing from EPIC and self-report. All parents will receive an automatic daily text in the morning with a link to report their daily activities with infants when they come to the NICU. All parents are signing up for daily texts for up to 20 weeks to complete daily activities report every day during their participation. All parents will also receive a weekly phone call from a research nurse from enrollment until discharge. M-MILK is primarily implemented by nurses or parents. **At baseline (T0)**, we will conduct the EPDS assessment, and collect parent characteristics,

buccal swab and fecal samples. For infants in the M-MILK group, the clinical team will place an order in EPIC for M-MILK to be provided starting on day 3 of life according to the M-MILK protocol. We will also schedule an in-person 30-minute M-MILK training session with parents in the M-MILK group, within 1 week of enrollment, provided by a research nurse. Parents in the M-MILK group are encouraged and supported to provide M-MILK when appropriate with the nurse's supervision. **At 34 weeks PMA (T1)**, we will collect PBQ, buccal swab and fecal samples. **At oral feeding initiation (T2)**, we will collect the EFS, and NCAST-Feeding video recording. Day of initiation of oral feeding is defined as the first of at least two consecutive days when the infant is able to orally consume ≥ 10% of their prescribed feedings. Infants will be fed by nurses, speech therapist, or PI. We will implement the same feeding protocol, e.g., side-lying and swaddling during feeding. **During the week of planned discharge (T3)**, we will conduct EPDS, PSS: NICU, PDR, PBQ, Pumping and Breastfeeding, NMI, NAPI, and EFS assessments. We will collect NCAST-Feeding video recording, buccal swab and fecal samples. For the NCAST-Feeding video recording, both parents and infants will be recorded. Parents will also complete the M-MILK Acceptability Questionnaire at T3. For EFS and NAPI assessments, we will video record the assessments, when possible, to obtain videos for inter-rater reliability. **At 2 months CA (T4)**, we will send parents the fecal and buccal sample collection kits via USPS mail service. We will also conduct a phone/Zoom interview to complete the EPDS, PBQ, ASQ-3, and NeoEAT assessments. During the phone/Zoom interview, we will also provide parents instructions on how to collect buccal and fecal samples and securely send them back to us.

VIII. Safety Monitoring Plan

a. Potential Risk

IRB oversight and approval will be based on 45CFR 46 HHS criteria. We anticipate our study will be determined to be no more than "minimal risk". The M-MILK intervention's safety has been demonstrated in infants as young as 22 weeks GA. According to the M-MILK protocol, nurses or parents will carefully observe infants' cues and responses during the M-MILK intervention and stop the administration if infants do not engage or exhibit any signs of distress. Thus, there is minimal risk associated with this protocol. Every effort will be made to minimize the risks. There may be risks associated with administration of M-MILK and collecting buccal swab samples including gagging and minor discomfort. Of note, these risks are no more expected risks to infants than what they might experience during a typical day, routine oral care, or feeding. Nurses will document infants' responses or any unexpected and serious adverse events to M-MILK intervention in the M-MILK Documentation paper sheet. A research nurse will review the documentation and report to PI if there are any unexpected and serious adverse events. For the NAPI assessment, we will perform maneuvers such as partially removing swaddle blanket, gentle stretching/bending of arms and legs, gently picking up, and stimulating vision and hearing with soft rattle sounds and talking voices. These maneuvers may pose minimal risks such as feeling cold, and mild discomfort due to stretching/bending/picking up, or noises. Of note, these risks are no more expected risks to infants than what they might experience during a typical day, routine care, or physical exam. Additionally, this research also carries a minimal risk of loss of data confidentiality/security. We also recognize that there are unique risks related to loss of confidentiality regarding a participant's epigenetic and microbiome data; however, we consider this risk to be rare.

b. Adequacy of Protection Against Risks

i. Informed Consent and Assent

The potential risks will be presented to the parents prior to enrollment. If parents are interested in having their preterm infants participate in the study, written informed consent will be obtained. Alternatively, the potential preterm infant will receive standard care if the parents choose to not enroll in the study. Premature infants, by virtue of their age cannot give consent or assent and therefore will not give assent or participate in the informed consent process.

ii. Protections Against Risk

1. Risks Related to Study Procedure

During the study, if an infant becomes clinically unstable and/or develops new diagnosis, and no longer meet the inclusion criteria, we will remove the infant from the research protocol. The M-MILK intervention's safety has been demonstrated in infants as young as 22 weeks GA. According to the M-MILK protocol, nurses or parents will carefully observe infants' cues and responses during the M-MILK intervention and stop the administration if infants do not engage or exhibit any signs of distress. When collecting the buccal swab samples and administering NAPI, we will make every effort to be as gentle as possible and omit any item(s) that may be causing discomfort to infants. If parents report a score of 12 or above on the EPDS, we will make referrals to the NICU Social Worker. If parents report "yes, quite often" or "sometimes" to the question "the thought of harming myself has occurred to me" on the EPDS, we will refer the parent to the local Emergency Department immediately. For the PBQ, parents scoring ≥ 26, (indicating risk of general bonding disorders) or ≥ 40 (indicating risk of severe bonding disturbances) should seek medical attention. For the PBQ, parents scoring "rarely", "sometimes", "quite often", "very often", or "always" for item 18 "I have done harmful things to my baby" or item 24 "I feel like hurting my baby" indicates risk of child abuse. In this case, it is our responsibility to report it to the state's child protective agency. If the infant is in immediate danger, the research staff will be instructed to inform the parents of their immediate concerns for the infant's safety, report the incident using a cellular phone, and call 911 until appropriate officials arrive. The PI will be informed of the family's condition and always be available by phone or in person for consultation and instruction. As part of the training of our research team, the team will review content related to reporting of child neglect and abuse.

2. Risks related to Data Confidentiality

Risks associated with loss of electronic data confidentiality/security will be mitigated through the utilization of secure electronic database (REDCap) and research network drive process to ensure that only authorized research personnel have access to the research data. The original signed informed consent documents will be kept in a locked cabinet at LUC CTRE. All infants will be assigned a study ID number. All of the data will be identified only by a study ID number. All discussions of specific research participants in our research team meetings will use this number rather than names. Electronic data will be encrypted with a password known and viewed only to the research staff. The password will be changed every six months.

The M-MILK Documentation paper sheets will have the infant's name on it and is available at the infant's bedside for nurses to document M-MILK. The completed sheets will be collected daily by a NICU charge nurse and stored in a locked cabinet on the unit. A research team member will pick up the completed sheets daily, and stored them in a locked cabinet in at LUC CTRE (Maywood, IL) for data entry. Upon completion of data entry into REDCap, the completed sheets will be shredded immediately.

Buccal swab and fecal samples will be labeled only with study ID numbers and collection date, and stored in a locked cabinet in a locked laboratory at LUC CTRE (Maywood, IL). We will securely transport buccal swab and fecal samples to Rush (Chicago, IL) using biosafety transfer containers and via car.

Video recordings will be downloaded and stored on a secure research network drive, hosted by LUC. Infant's and parent's face will be visible in the videos as we need to observe their interactions and infant's feeding skills. However, we will not include any identifiers such as name or date in the videos. The video files will be labeled only with study ID numbers and recorded date.

De-identifiable data will be shared with University of Illinois at Chicago Research Informatics Core. All data; e.g., raw sequence data, processed data and sample mapping; will be transmitted between systems via encrypted transport protocols, i.e. HTTPS or SFTP. All desktop and laptop computers are protected by strong password authentication with whole disk encryption enabled. Remote servers are protected by network firewalls, intrusion detection software, and either bastions or two factor authentication for any remote logins. Any passwords on the remote systems are subject to strong password requirements, i.e., minimum password length, combinations of different character types (capital letters, lowercase letters, numbers, special characters), and are not typical English words.

Biospecimens are materials that come from infants that may include blood, tissue, urine, bone marrow, saliva, cells, etc. In this study, we will collect cheek tissue and stool. Most biospecimens contain DNA. We will not use biospecimens collected as a part of this study for whole genome sequencing. However, we will use biospecimens collected as a part of this study to sequence a short portion of the genes of interest that starts gene transcription found at the beginning of the genes. The de-identified sequence data will be submitted to online government data repositories per National Institute of Health requirements. All the sources of the sample are de-identified and infants' individual identity will not be revealed.

De-identified data, photos and/or selected brief segments of videos from this study may be shared with others for research/educational purposes in reports, scientific papers, research/educational meetings, and/or research/educational websites. De-identified data, photos and/or selected brief segments of videos from this study may also be shared on research/educational social media platforms. We will remove or code any personal identifiable information that could identify participants before data are shared with other researchers to ensure that no one will be able to identify participants from the information we share. De-identified data, the photos and/or selected brief segments of videos will be stored indefinitely. Informed consents and video recordings will be securely shredded/deleted after all related data are published or up to 5 years. Buccal swab and fecal samples will be stored and securely destroyed after all related data are published or up to 5 years.

If any data are lost or stolen, the PI will immediately notify the LUC Information Services, at 773-508-4487 and will notify the LUC IRB of any unusual occurrence.

The results of this research study may be published in a journal for the purpose of advancing medical knowledge. You will not be identified by name or by any other identifying information in any publication or report about this research. When we prepare our reports or publications, we will summarize the results of the research in a manner that will not reveal infants' identity. In addition, no names will be used in any report of this study. We may want to use photos and/or brief segments of the videos to illustrate infants' feeding skills and behaviors in reports, scientific papers, or educational meetings. We will obtain parents' written permission for use of evaluation photos and videos for educational purposes.

3. Report of child neglect and abuse to the appropriate authority

Researchers and professionals who work with families with children under the age of 18 are responsible for the welfare of children. For the PBQ, parents scoring "rarely", "sometimes", "quite often", "very often", or "always" for item 18 "I have done harmful things to my baby" or item 24 "I feel like hurting my baby" indicates risk of child abuse. If the research team suspects an infant has been physically or emotionally abused or neglected, following the guidelines set by the state during study evaluations, it is our responsibility to report it to the state's child protective agency. If the infant is in immediate danger, the research staff will be instructed to inform the parents of their immediate concerns for the infant's safety, report the incident using a cellular phone, and call 911 until appropriate officials arrive. The PI will be informed of the family's condition and always be available by phone or in person for consultation and instruction. As part of the training of our research team, the team will review content related to reporting of child neglect and abuse.

iii. Vulnerable Subjects

This research study is intended to evaluate an intervention for a specific group of preterm infants, those born between 22 to 33 weeks GA. It is essential that infants under one year of age are included. Older children are excluded because the study is evaluating preterm infants only. Our research team has expertise for working with preterm infants. For example, Dr. Griffith has more than 12 years of experience working with preterm infants and their families. Dr. White-Traut has more than 10 years of clinical experience and 40 years of research experience conducting numerous research projects, including H-HOPE intervention with neonates and their mothers. The infants will be cared for in the NICU as part of their hospitalization. The team will follow the U.S. Department of Health and Human Services regulatory requirements for parental permission and child assent for research involving children. Preterm infants, by virtue of their age cannot give consent or assent and therefore will not give assent or participate in the informed consent process.

IX. Data Management and Analysis Plan

Strict confidentiality of data files will be maintained. Data management will be facilitated using REDCap. SAS (v 9.4) will be used for all statistical procedures and analyses. Levels of missingness, variable distributions, and outliers will be examined throughout data collection for quality assurance as well as prior to statistical modeling. We will calculate descriptive statistics for all variables of interest to describe the variability of study variables, and conduct sample size estimation for the planned R01 application. Comparisons will be made using an intent-to-treat approach with participants analyzed in the groups to which they were randomized. Feasibility will be assessed with respect to enrollment, adherence, acceptability, and safety. We will calculate the percentage of infants who (1) were eligible, approached, consented, enrolled, and randomized over the recruitment period, (2) were retained for the duration of the study, (3) completed all inpatient data collection, and (4) completed post-discharge follow-ups. To determine the adherence of M-MILK, we will calculate the daily numbers of completed M-MILK and the daily numbers of expected M-MILK implementation (numbers of hands-on care episodes before full gavage feedings) to obtain the daily percentage of M-MILK completion. We will summarize findings from M-MILK Acceptability Questionnaire by nurses and parents. We will also summarize findings from M-MILK EPIC charting regarding infant's responses and any adverse events to monitor for the safety of M-MILK.

X. Study Timeline

Table 2. The proposed pilot study will require 1 year to complete. During the first 3 months, we will hire and train the team members. We anticipate enrolling 2 infants per month for 9 months. An additional 3 months will be required to complete the intervention and collect study data. Data entry and analyses will be ongoing. We will spend 6 months to complete data analyses. An additional 6 months will be needed to prepare the final report and disseminations.

Table 2. Study Timeline

Activity/Month	Month 1-3	Month 3-12	Month 12-15	Month 15-21	Month 21-27
Preparation & Staff Training	**X**				
Recruitment & Enrollment		**X**			
Data Collection		**X**	**X**		
Data Entry & Analyses		**X**	**X**	**X**	
Final Reports & Dissemination				**X**	**X**

XIII. Literature Cited

1. Barfield WD. Public Health Implications of Very Preterm Birth. *Clin Perinatol.* Sep 2018;45(3):565-577.
2. Pickler RH, Wetzel PA, Meinzen-Derr J, Tubbs-Cooley HL, Moore M. Patterned feeding experience for preterm infants: study protocol for a randomized controlled trial. *Trials.* Jun 4 2015;16:255.
3. Griffith T, White-Traut R, Janusek LW. A Behavioral Epigenetics Model to Predict Oral Feeding Skills in Preterm Infants. *Adv Neonatal Care.* Oct 2020;20(5):392-400.
4. Moschino L, Duci M, Fascetti Leon F, et al. Optimizing Nutritional Strategies to Prevent Necrotizing Enterocolitis and Growth Failure after Bowel Resection. *Nutrients.* Jan 24 2021;13(2).
5. Tang MN, Adolphe S, Rogers SR, Frank DA. Failure to Thrive or Growth Faltering: Medical, Developmental/Behavioral, Nutritional, and Social Dimensions. *Pediatr Rev.* Nov 2021;42(11):590-603.
6. Goh GL, Lim CSE, Sultana R, De La Puerta R, Rajadurai VS, Yeo KT. Risk Factors for Mortality From Late-Onset Sepsis Among Preterm Very-Low-Birthweight Infants: A Single-Center Cohort Study From Singapore. *Front Pediatr.* 2021;9:801955.
7. Bergman NJ. Birth Practices: Maternal-Neonate Separation as a Source of Toxic Stress. *Birth Defects Res.* Sep 1 2019;111(15):1087-1109.
8. de Magalhães-Barbosa MC, Prata-Barbosa A, da Cunha A. Toxic Stress, Epigenetics and Child Development. *J Pediatr (Rio J).* Mar-Apr 2022;98 Suppl 1(Suppl 1):S13-s18.
9. National Association of Neonatal Nurses. Trauma-Informed Care in the NICU. 2022.

10. Malin K, Gondwe KW, Fial AV, et al. A scoping review of epigenetic alterations in premature infants secondary to early toxic stress exposure. *Nursing Research.* Accepted 2022.
11. Lester BM, Marsit CJ, Giarraputo J, Hawes K, LaGasse LL, Padbury JF. Neurobehavior Related to Epigenetic Differences in Preterm Infants. *Epigenomics.* Oct 2015;7(7):1123-1136.
12. Zhao T, Griffith T, Zhang Y, et al. Early-life factors associated with neurobehavioral outcomes in preterm infants during NICU hospitalization. *Pediatr Res.* Mar 25 2022.
13. Provenzi L, Guida E, Montirosso R. Preterm Behavioral Epigenetics: A Systematic Review. *Neurosci Biobehav Rev.* Jan 2018;84:262-271.
14. Cowan CSM, Stylianakis AA, Richardson R. Early-life stress, microbiota, and brain development: probiotics reverse the effects of maternal separation on neural circuits underpinning fear expression and extinction in infant rats. *Dev Cogn Neurosci.* Jun 2019;37:100627.
15. D'Agata AL, Wu J, Welandawe MKV, Dutra SVO, Kane B, Groer MW. Effects of early life NICU stress on the developing gut microbiome. *Dev Psychobiol.* Jul 2019;61(5):650-660.
16. Groer MW, Miller EM, D'Agata A, et al. Contributors to Dysbiosis in Very-Low-Birth-Weight Infants. *J Obstet Gynecol Neonatal Nurs.* May 2020;49(3):232-242.
17. Henderickx JGE, Zwittink RD, van Lingen RA, Knol J, Belzer C. The Preterm Gut Microbiota: An Inconspicuous Challenge in Nutritional Neonatal Care. *Front Cell Infect Microbiol.* 2019;9:85.
18. Liu X, Cong X, Li G, Maas K, Chen K. Multivariate log-contrast regression with sub-compositional predictors: Testing the association between preterm infants' gut microbiome and neurobehavioral outcomes. *Stat Med.* Feb 10 2022;41(3):580-594.
19. Lu J, Claud EC. Connection Between Gut Microbiome and Brain Development in Preterm Infants. *Dev Psychobiol.* Jul 2019;61(5):739-751.
20. Janusek LW, Tell D, Gaylord-Harden N, Mathews HL. Relationship of Childhood Adversity and Neighborhood Violence to a Proinflammatory Phenotype in Emerging Adult African American Men: An Epigenetic Link. *Brain Behav Immun.* Feb 2017;60:126-135.
21. Janusek LW, Tell D, Mathews HL. Epigenetic Perpetuation of the Impact of Early Life Stress on Behavior. *Curr Opin Behav Sci.* 2019;28C:1-7.
22. Cong X, Judge M, Xu W, et al. Influence of Feeding Type on Gut Microbiome Development in Hospitalized Preterm Infants. *Nurs Res.* Mar/Apr 2017;66(2):123-133.
23. Xu W, Judge MP, Maas K, et al. Systematic Review of the Effect of Enteral Feeding on Gut Microbiota in Preterm Infants. *J Obstet Gynecol Neonatal Nurs.* May 2018;47(3):451-463.
24. Lester BM, Conradt E, LaGasse LL, Tronick EZ, Padbury JF, Marsit CJ. Epigenetic Programming by Maternal Behavior in the Human Infant. *Pediatrics.* Oct 2018;142(4).
25. Provenzi L, Brambilla M, Scotto di Minico G, Montirosso R, Borgatti R. Maternal Caregiving and DNA Methylation in Human Infants and Children: Systematic Review. *Genes Brain Behav.* Mar 2020;19(3):e12616.
26. Álvarez MJ, Fernández D, Gómez-Salgado J, Rodríguez-González D, Rosón M, Lapeña S. The Effects of Massage Therapy in Hospitalized Preterm Neonates: A Systematic Review. *Int J Nurs Stud.* Apr 2017;69:119-136.
27. Wetzel CM, Davis L, Grohler N, et al. A Quality Improvement Project to Improve the Use of Mother's Own Milk (MOM) With Precision Oropharyngeal Therapy. *Adv Neonatal Care.* Apr 2020;20(2):E19-e30.
28. O'Rouke B, Fuller K, Parker LA, Moore TA. Provision of Positive Oral Experiences for Premature Infants by Offering Milk Drops: A Clinical Practice Change Initiative *Adv Neonatal Care.* 2022.
29. Abd-Elgawad M, Eldegla H, Khashaba M, Nasef N. Oropharyngeal Administration of Mother's Milk Prior to Gavage Feeding in Preterm Infants: A Pilot Randomized Control Trial. *JPEN J Parenter Enteral Nutr.* Jan 2020;44(1):92-104.
30. Muelbert M, Lin L, Bloomfield FH, Harding JE. Exposure to the Smell and Taste of Milk to Accelerate Feeding in Preterm Infants. *Cochrane Database Syst Rev.* Jul 16 2019;7(7):Cd013038.
31. Moore CG, Carter RE, Nietert PJ, Stewart PW. Recommendations for planning pilot studies in clinical and translational research. *Clin Transl Sci.* Oct 2011;4(5):332-337.
32. Cox JL, Holden JM, Sagovsky R. Detection of postnatal depression. Development of the 10-item Edinburgh Postnatal Depression Scale. *The British journal of psychiatry : the journal of mental science.* Jun 1987;150:782-786.

33. Park SH, Kim JI. Predictive validity of the Edinburgh postnatal depression scale and other tools for screening depression in pregnant and postpartum women: a systematic review and meta-analysis. *Arch Gynecol Obstet.* May 2023;307(5):1331-1345.
34. Miles MS, Funk SG, Carlson J. Parental Stressor Scale: neonatal intensive care unit. *Nurs Res.* May-Jun 1993;42(3):148-152.
35. Kubicka Z, Zahr E, Clark P, Williams D, Berbert L, Arzuaga B. Use of an internet camera system in the neonatal intensive care unit: parental and nursing perspectives and its effects on stress. *J Perinatol.* Aug 2021;41(8):2048-2056.
36. Fischer SL. Modification and Evaluation of the Late-preterm Screening and Multidisciplinary Risk Assessment and Parent Activation Scale. South Dakota, USA: South Dakota State University; 2013.
37. Korner AF, Stevenson DK, Kraemer HC, et al. Prediction of the Development of Low Birth Weight Preterm Infants by a New Neonatal Medical Index. *J Dev Behav Pediatr.* Apr 1993;14(2):106-111.
38. Cong X, Wu J, Vittner D, et al. The impact of cumulative pain/stress on neurobehavioral development of preterm infants in the NICU. *Early Hum Dev.* May 2017;108:9-16.
39. Newnham CA, Inder TE, Milgrom J. Measuring Preterm Cumulative Stressors Within the NICU: The Neonatal Infant Stressor Scale. *Early Hum Dev.* Sep 2009;85(9):549-555.
40. Smith GC, Gutovich J, Smyser C, et al. Neonatal Intensive Care Unit Stress is Associated with Brain Development in Preterm Infants. *Ann Neurol.* Oct 2011;70(4):541-549.
41. Gorzilio DM, Garrido E, Gaspardo CM, Martinez FE, Linhares MB. Neurobehavioral Development Prior to Term-Age of Preterm Infants and Acute Stressful Events During Neonatal Hospitalization. *Early Hum Dev.* Dec 2015;91(12):769-775.
42. Gabriel P, Formiga C, Linhares M. Early neurobehavioral development of preterm infants. *Psicologia.* 2013;26(1):202-211.
43. Constantinou JC, Adamson-Macedo EN, Mirmiran M, Ariagno RL, Fleisher BE. Neurobehavioral assessment predicts differential outcome between VLBW and ELBW preterm infants. *J Perinatol.* Dec 2005;25(12):788-793.
44. Snider L, Tremblay S, Limperopoulos C, Majnemer A, Filion F, Johnston C. Construct validity of the Neurobehavioral Assessment of Preterm Infants. *Physical & occupational therapy in pediatrics.* 2005;25(3):81-95.
45. Korner A, Brown J, Thom V, Constantinou J. *Neurobehavioral Assessment of the Preterm Infant revised*. 2nd ed. Van Nuys: Child Development Media, Inc. ; 2000.
46. Korner AF, Constantinou J, Dimiceli S, Brown BW, Jr., Thom VA. Establishing the reliability and developmental validity of a neurobehavioral assessment for preterm infants: a methodological process. *Child development.* Oct 1991;62(5):1200-1208.
47. Korner AF, Kraemer HC, Reade EP, Forrest T, Dimiceli S, Thom VA. A methodological approach to developing an assessment procedure for testing the neurobehavioral maturity of preterm infants. *Child development.* Dec 1987;58(6):1478-1487.
48. Shuffrey LC, Firestein MR, Kyle MH, et al. Association of Birth During the COVID-19 Pandemic With Neurodevelopmental Status at 6 Months in Infants With and Without In Utero Exposure to Maternal SARS-CoV-2 Infection. *JAMA Pediatr.* Jun 1 2022;176(6):e215563.
49. Lipkin PH, Macias MM. Promoting Optimal Development: Identifying Infants and Young Children With Developmental Disorders Through Developmental Surveillance and Screening. *Pediatrics.* Jan 2020;145(1).
50. Agarwal PK, Xie H, Sathyapalan Rema AS, et al. Evaluation of the Ages and Stages Questionnaire (ASQ 3) As a Developmental Screener at 9, 18, and 24 Months. *Early Hum Dev.* Aug 2020;147:105081.
51. Thoyre SM, Pados BF, Shaker CS, Fuller K, Park J. Psychometric Properties of the Early Feeding Skills Assessment Tool. *Adv Neonatal Care.* Oct 2018;18(5):E13-E23.
52. Pados BF, Thoyre SM, Estrem HH, Park J, McComish C. Factor Structure and Psychometric Properties of the Neonatal Eating Assessment Tool-Breastfeeding. *J Obstet Gynecol Neonatal Nurs.* May 2018;47(3):396-414.

53. Pados BF, Thoyre SM, Estrem HH, Park J, McComish C. Factor Structure and Psychometric Properties of the Neonatal Eating Assessment Tool-Bottle-Feeding (NeoEAT-Bottle-Feeding). *Adv Neonatal Care.* Jun 2018;18(3):232-242.
54. Arianas EA, Rankin KM, Norr KF, White-Traut RC. Maternal weight status and responsiveness to preterm infant behavioral cues during feeding. *BMC Pregnancy Childbirth.* Apr 11 2017;17(1):113.
55. Farel AM, Freeman VA, Keenan NL, Huber CJ. Interaction between high-risk infants and their mothers: the NCAST as an assessment tool. *Res Nurs Health.* Apr 1991;14(2):109-118.
56. Jameson M, Fehringer K, Neu M. Comparison of two tools to assess dyad feeding interaction in infants with gastroesophageal reflux disease. *J Spec Pediatr Nurs.* Jan 2018;23(1).
57. Letourneau NL, Tryphonopoulos PD, Novick J, Hart JM, Giesbrecht G, Oxford ML. Nursing Child Assessment Satellite Training Parent-Child Interaction Scales: Comparing American and Canadian Normative and High-Risk Samples. *J Pediatr Nurs.* May-Jun 2018;40:47-57.
58. White-Traut R, Norr KF, Fabiyi C, Rankin KM, Li Z, Liu L. Mother-infant interaction improves with a developmental intervention for mother-preterm infant dyads. *Infant Behav Dev.* Dec 2013;36(4):694-706.
59. Whitfield KC, Ventura AK. Exploration of Responsive Feeding During Breastfeeding Versus Bottle Feeding of Human Milk: A Within-Subject Pilot Study. *Breastfeed Med.* Sep 2019;14(7):482-486.
60. McFadden VC, Shalaby RE, Iram S, et al. Hepatic Deficiency of the Pioneer Transcription Factor FoxA Restricts Hepatitis B Virus Biosynthesis by the Developmental Regulation of Viral DNA Methylation. *PLOS Pathog.* Feb 2017;13(2):e1006239.
61. Lester BM, Marsit CJ. Epigenetic Mechanisms in the Placenta Related to Infant Neurodevelopment. *Epigenomics.* Mar 1 2018;10(3):321-333.
62. Casavant SG, Cong X, Moore J, Starkweather A. Associations between preterm infant stress, epigenetic alteration, telomere length and neurodevelopmental outcomes: A systematic review. *Early Hum Dev.* Apr 2019;131:63-74.
63. Vaiserman AM, Koliada AK. Early-life adversity and long-term neurobehavioral outcomes: epigenome as a bridge? *Hum Genomics.* Dec 16 2017;11(1):34.
64. Hooper MJ, LeWitt TM, Pang Y, et al. Gut dysbiosis in cutaneous T-cell lymphoma is characterized by shifts in relative abundances of specific bacterial taxa and decreased diversity in more advanced disease. *J Eur Acad Dermatol Venereol.* Sep 2022;36(9):1552-1563.
65. Naqib A, Poggi S, Wang W, Hyde M, Kunstman K, Green SJ. Making and Sequencing Heavily Multiplexed, High-Throughput 16S Ribosomal RNA Gene Amplicon Libraries Using a Flexible, Two-Stage PCR Protocol. *Methods Mol Biol.* 2018;1783:149-169.
66. Walters W, Hyde ER, Berg-Lyons D, et al. Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Surveys. *mSystems.* Jan-Feb 2016;1(1).
67. Gihring TM, Green SJ, Schadt CW. Massively parallel rRNA gene sequencing exacerbates the potential for biased community diversity comparisons due to variable library sizes. *Environ Microbiol.* Feb 2012;14(2):285-290.
68. *R Package. Vegan: community ecology* [computer program]. Version; 2018.
69. Jackson A, Engen PA, Forsyth CB, et al. Intestinal Barrier Dysfunction in the Absence of Systemic Inflammation Fails to Exacerbate Motor Dysfunction and Brain Pathology in a Mouse Model of Parkinson's Disease. *Front Neurol.* 2022;13:882628.
70. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 2014;15(12):550.
71. Weiss S, Xu ZZ, Peddada S, et al. Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome.* Mar 3 2017;5(1):27.