Official Title: Somatosensory Modulation of Salivary Gene Expression and Oral Feeding in Preterm Infants
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Protocol

Preterm infants will be recruited at gestational age 24 $^{0/7}$-28 $^{6/7}$ weeks GA. Each infant will be randomized to receive either the PULSED NTrainer or SHAM intervention using a software random integer function in Minitab v.17 (www.minitab.com). As shown in Figure 1, infants assigned to the PULSED NTrainer group will receive a progressive dose of the pulsatile orocutaneous stimulation. Beginning at 30 weeks' PCA, these infants will receive 2 weeks of low-dose PULSED NTrainer stimulation (2 x 3-minute blocks) with a 1-minute stimulus 'off-period' between the stimulation blocks. This form of stimulation will be given simultaneous with tube feedings 3 times/day. The stimulus dose will then be increased over the next 2 weeks (3 x 3-minute blocks of PULSED NTrainer stimulation) with a 1-minute stimulus 'off-period' between the stimulation blocks, also given simultaneously with tube feedings 3 times/day. Preterm infants randomized to the SHAM condition will be given a regular silicone pacifier during tube feedings over the same time period.

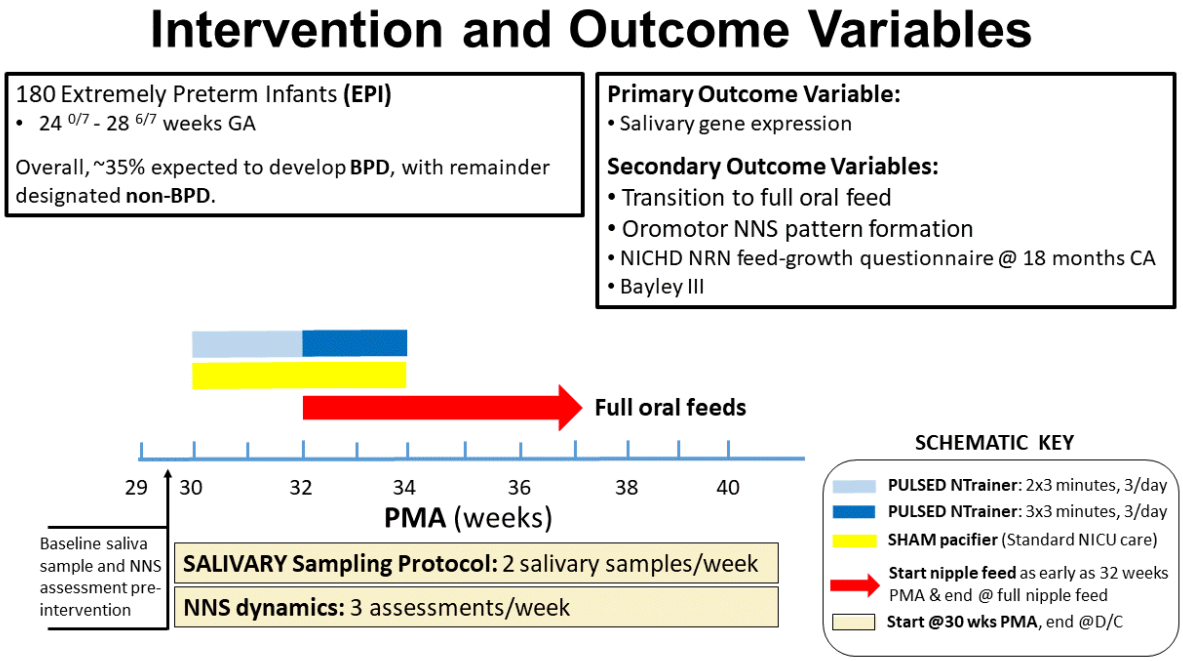

Figure 1. Somatosensory intervention plan for preterm infants along with the salivary sampling protocol and digitized measurements of non-nutritive suck progression is shown above. Primary (salivary gene expression) , and secondary (transition to full oral feed, oromotor NNS pattern formation, NICHD Neonatal Research Network feeding-growth questionnaire, and Bayley III screener at NICU follow-up) outcome variables are given.

Orocutaneous Stimulation Regimen

The NTrainer PULSED orocutaneous stimulus consists of a series of 6-cycle bursts which are delivered by a servo-controlled pneumatic amplifier (NTrainer System®) to the lumen of a standard silicone pacifier (e.g., WeeSoothie, or Soothie). These pneumatic bursts are frequency modulated (FM) from 2.8 to 1.6 Hz across the 6-cycle structure with a 2-second

pause period between bursts (Figure 2). Individual pressure cycles have a 31 millisecond (ms) rise/fall time to ensure a salient stimulus spectra with significant energy from DC-16 Hz. Frequency modulation is a physiologic feature of the non-nutritive suck (NNS) in preterm infants. A total of 34 bursts are presented in a 3-min block. A 1-minute rest period (no stimulation) occurs between stimulation blocks. Criteria for initiation of orocutaneous therapy include the following: 1) stable vital signs and not on continuous vasopressor medications; 2) tolerating enteral feeds in previous 48 hours; and 3) not intubated and mechanically ventilated. If the infant is on nasal intermittent positive pressure ventilation, continuous positive airway pressure or nasal cannula >2 liters per minute, then the FiO2 must be <40%.

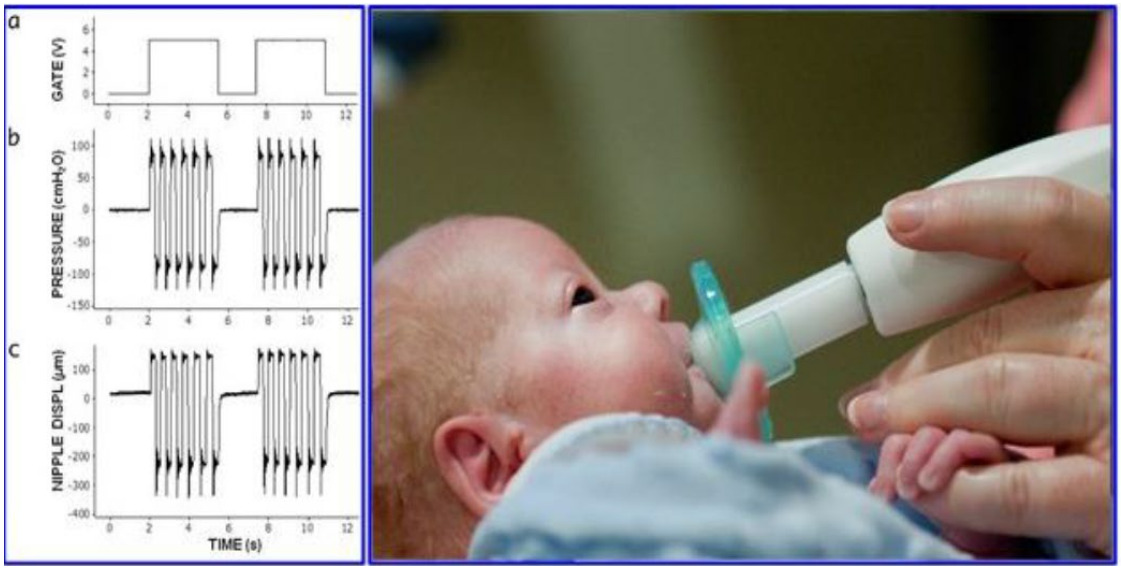

Figure 2. Preterm infant receiving PULSED NTrainer stimulation during gavage feeding in the NICU. NG tube is placed through the left nares (not visible). Pneumatic stimulus control signals and output through the pacifier nipple are shown in the left panel. a) voltage-controller gate signal, b) intraluminal pressure (inside) the nipple, and c) mechanical displacement at the nipple cylinder wall.

NNS Assessment and Automated NNS Digital Signal Processing and Feature Extraction

In addition to the oral stimulation interventions (PULSED NTrainer vs. SHAM pacifier), all study infants will be assessed 3 times/week (e.g., Mon/Wed/Fri) for NNS performance. The NTrainer System® will be used in '*assessment mode*' to record the compression dynamics of NNS during a 3-minute session to immediately precede a tube feeding that is not associated with an intervention condition.

The most active 2-minute period of NNS behavior based on suck cycle count is automatically extracted from each suck assessment data file using an automated waveform feature extraction algorithm on the NTrainer System®. The NNS pressure waveform is band-pass filtered (0.5-20 Hz) to remove low frequency offsets due to tongue/jaw posturing and thermal drift associated with oral contact on the pacifier bulb, and to remove high-frequency jitter. Pressure peaks > 1.6 cm H2O are subjected to feature extraction criteria, including suck cycle symmetry, cycle duration, and burst identification defined as two or more NNS events occurring within 1200 ms.

This algorithm permits objective identification of NNS burst activity distinct from non-NNS mouthing compressions or tongue thrusts against the pacifier. Four measures will be objectively extracted from the indexed records of suck compression, including (1) NNS Bursts/min where an individual burst includes 2 or more suck cycles, (2) NNS Cycles/min, (3) NNS Spatiotemporal Index, and (4) NNS compression pressure (cm H_2O).

PO Feeding Advancement

Preterm infants will advance on a standardized cue-based feeding schedule utilized by each site NICU, known as Infant Driven Feeding® that will lead to full nipple feeds. This standardization of oral feeding advancements across sites will limit confounders that may ultimately skew the data.

Preterm infant participant data will be managed with our Neonatal Oromotor Database, a custom software developed in the Barlow laboratory specifically for NTrainer studies in the NICU. This database software is compatible with MS WIN operating systems, and is password-protected and coded as an executable for MS ACCESS 2013. This software provides a paperless, efficient system for NICU study personnel to log daily information including GA, growth parameters, medications, oxygen requirement, feeding history, medical procedure log, comments, as well as NTrainer/SHAM and salivary sampling dates on enrolled infants.

Saliva Collection, Processing, and Gene Expression Analysis

Saliva Collection: Saliva samples will be collected from all enrolled infants on the first day prior to receiving either the SHAM or PULSED NTrainer intervention. This sample will serve as our baseline gene expression profile. Samples will then be obtained twice per week (~3 day intervals) up to the time of achievement of full oral feeds or discharge home from the hospital with either a nasogastric tube (NG) or gastronomy tube.

Saliva samples at each site will be collected with techniques that have been optimized and validated through the Maron laboratory. Briefly, saliva samples will be collected with a 1 mL syringe, end caps removed and attached to low wall suction. Saliva will be collected for approximately 20 seconds and immediately stabilized in 500 µL of Qiagen's RNA Protect Saliva to halt gene expression changes, inhibit destructive RNases, and limit microbial overgrowth. Samples will be vortexed briefly and frozen at -20°C. Once frozen, samples are stable for up to 18 months prior to the need for total RNA extraction. Thus, samples from Nebraska and Santa Clara will be shipped once a month on ice overnight to the Maron laboratory at the Mother Infant Research Institute at Tufts Medical Center in Boston, MA for processing.

Generation of a Saliva Biobank: One additional saliva sample will be obtained each week from all enrolled subjects to generate a biobank repository. The rationale for this approach is twofold. First, it is possible that during shipment or processing, salivary RNA may be destroyed and not be of sufficient quality to incorporate into the study. Prior studies from this lab have demonstrated a 10% failure rate of gene amplification in saliva samples. Banking an additional sample each week from study subjects will ensure that we will limit loss of data points. Second, we anticipate that there are informative salivary biomarkers of oral feeding maturation that have yet to be identified. By generating a biobank of additional saliva samples that may be retrospectively interrogated on either a microarray gene expression platform or with RNASeq, we will have the potential to discover novel hypotheses about gene-gene interactions and/or transcriptional regulatory processes related to oral feeding in the preterm population.

Total RNA Extraction: Salivary samples will extracted for total RNA using established protocols optimized in the Maron laboratory. RNA extraction will occur with the QIAGEN RNAProtect Saliva Mini kit per manufacturer's instructions. On column DNase treatment will be performed for each sample to eliminate genomic DNA contamination. Samples that are part of the biobank will be frozen at -80°C pending need for future analysis. Samples that will be used for RT-qPCR experiments will first undergo cDNA conversion with the Life Technologies SuperScript® Vilo™ kit per manufacturer's instructions. cDNA will be stored at -80°C in the Mother Infant Research Institute at Tufts Medical Center pending gene expression analysis.

Gene Expression Analysis: For the purpose of this study, we will interrogate each sample for nine genes, six target genes of interest and three reference genes for quality control and potential normalization of gene expression data. Genes to be analyzed in this study have been previously shown to be directly linked to oral feeding in the newborn and include *CDH13, FOXP2, NPHP4, NPY2R, PLXNA1,* and *WNT3.* The three reference genes include *GAPDH*, *HPRT1*, and *YWHAZ*, all of which have been shown to maintain stable gene expression across advancing PCA. All cDNA samples will undergo a targeted pre-amplification with a custom TaqMan® PreAmp Master Mix for all nine genes. This targeted approach to amplification will ensure that only those genes of interest will be amplified and not all genes across the transcriptome which may introduce bias. Preamplified cDNA will then undergo PCR with TaqMan® Fast Advance Master Mix on the Life Technologies Quant Studio™ 7 Flex Real-Time PCR System. This instrument is housed with the Mother Infant Research Institute at Tufts Medical Center and is an advanced state-of-the-art PCR machine and software system. All efforts will be made to adhere to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines for this study. All samples will be run in duplicate with appropriate positive and negative controls.

Initial Gene Expression Analyses: All gene expression data will be normalized on the Quant Studio™ 7 Flex Real-Time PCR System. Only samples that have successful amplification of all three reference genes will be considered in the analysis. Previously, genes have been informative in a binary fashion (+/- expression). However, for the purpose of this study, we will be prepared to calculate relative gene expression of each target gene with the delta delta cycle threshold ($\Delta\Delta$Ct) method. In accordance to the MIQE guidelines, we will use the three reference genes for normalization and determine a geometric mean of their Ct in each sample to calculate relative gene expression. Normalized date will then be given to our statistician and bioinformatic collaborator for analysis.

Neurodevelopmental Follow-Up

Each center site in this study has a Neonatal Follow-Up Clinic that performs neurodevelopment testing on infants from discharge up to three years of life by certified examiners as part of standard of care. Thus, developmental follow-up data will be available from all study subjects, regardless of intervention, and free of charge. As part of standard of care, each site will complete the Bayley Scales of Infant and Toddler Development® 3rd Edition, on preterm infants at 18 to 24 months' corrected age (CA). The primary subtests of interest include *Fine Motor* (prehension, motor speed and planning, perceptual-motor integration, reaching, grasping, and object manipulation), *Gross Motor* (dynamic movement [i.e., walking, jumping, running, stairs, etc.], motor planning, balance, and perceptual-motor integration), *Cognition* (puzzle assembly, object completion, means-ends manipulation, representational play, counting, and matching colors), and *Language* (receptive and expressive language). These data will be recorded,

analyzed, and correlated with feeding outcomes in the neo-and post-natal period, gene expression data, and intervention status. In addition, growth parameters including head circumference, length and weight will be recorded.

Feeding behavior plays a significant role in neurodevelopmental outcomes. Recent findings from the NICHD Neonatal Research Network (NRN) indicated at 18 months' CA, premature infants with a history of feeding difficulties are more likely to have language delay. Neuromotor status and days on mechanical ventilation are important risk factors associated with these outcomes. A follow-up of feeding status will be completed when our study infants reach 18 months' CA using the NICHD NRN 18-month Feeding-Growth-Nutrition Questionnaire. This questionnaire includes a simple checklist format about medical history since the NICU (re-hospitalization, primary cause, time period, LOS, time in ICU), medications, seizures, supplemental oxygen and respiratory monitoring, oromotor skills (independent feeds, dependent oral feeds, limited oral feeding, no oral feeding), NG or TPN feeds, feeding behaviors (aversion, swallowing-dysphagia), aspiration (food down windpipe, choking), spit-ups, high calorie supplements, oral diet texture (thin vs thickened liquids, soft solids, table food), and surgical operations. Completion time by the parent is 15 minutes or less. The feeding questionnaire and a pre-posted return envelope will be mailed to the parent when a given study infant attains 17.5 months of age. A cover letter will accompany the 3-page questionnaire explaining that a nurse/study specialist from this research project will be available to assist with the questionnaire.