Somatosensory Modulation of Salivary Gene Expression and Oral Feeding in Preterm Infants

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Disclosures

- I have no financial disclosures or conflict of interests.
Significance

- In 2019, the Centers for Disease Control reported that 1 in 10 infants (~450,000) were born prematurely in the US
  - Defined as <37 weeks’ gestation

- 15 million babies are born prematurely worldwide

- Dated health economic data (2007) estimated that prematurity costs the US health care $26 billion/year

- Revised cost estimate of prematurity on the US health care system is $34.2 billion/yr

Clinical Dilemma: Oral Feeding

- **Majority** of these premature infants do not have the developmental maturity to successfully and safely feed by mouth.

- Infants must learn to orally feed prior to discharge from the NICU in accordance with AAP guidelines.

- Achievement of oral feeding competency is a major determinant of length of stay and a significant medical care cost driver.
Complexities of Oral Feeding

- Oral feeding competency relies upon the maturation and coordination of:
  
  \[ \text{Suck} \rightarrow \text{Swallow} \rightarrow \text{Breathe} \]

- However, the ability to feed is also driven by:

  **Senses:**

  **Gut – Brain Axis:**

  **Neurodevelopment:**

  - 25 weeks
  - 35 weeks
  - 40 weeks
Oral Feeding

• Oral feeding difficulties are not homogeneous
  – This is not a one size fits all model

• Infants may lag in one developmental system limiting their ability to feed.
  – Sensory integration, hunger signaling, oral motor control

• Biological variability also impacts feeding success
  – Sex, post-menstrual age, race, ethnicity
Impact of Sex

• Multicenter retrospective analysis of a prospective cohort of moderately preterm infants admitted to an NICHD NRN hospital

• Primary Outcomes: Post menstrual age at full oral feeding and at discharge home

• Subjects: 6,146 infants born between 29-33 weeks’ gestation between January 2012-November 2013

Brumbaugh et al. for the NRN, 2018 Early Hum Dev
# Delayed Oral Feeding Maturation

<table>
<thead>
<tr>
<th>Explanatory (independent) variable</th>
<th>Estimate</th>
<th>95% Confidence limits</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA at first feed (in weeks)</td>
<td>4.49</td>
<td>4.13</td>
<td>4.85</td>
</tr>
<tr>
<td>Birth weight (per 100 g)</td>
<td>-0.46</td>
<td>-0.51</td>
<td>-0.40</td>
</tr>
<tr>
<td>SGA</td>
<td>0.91</td>
<td>0.24</td>
<td>1.58</td>
</tr>
<tr>
<td>Male</td>
<td>1.31</td>
<td>0.87</td>
<td>1.76</td>
</tr>
<tr>
<td>Surfactant exposure</td>
<td>2.43</td>
<td>1.87</td>
<td>3.00</td>
</tr>
<tr>
<td>PDA requiring treatment</td>
<td>3.37</td>
<td>0.93</td>
<td>5.81</td>
</tr>
<tr>
<td>Black race</td>
<td>-1.62</td>
<td>-2.44</td>
<td>-0.80</td>
</tr>
<tr>
<td>Multiple gestation</td>
<td>0.59</td>
<td>0.09</td>
<td>1.10</td>
</tr>
<tr>
<td>Human milk in the first 28 days</td>
<td>0.77</td>
<td>0.11</td>
<td>1.44</td>
</tr>
</tbody>
</table>

Brumbaugh et al. for the NRN, 2018 Early Hum Dev
In Utero Differences

- Sex specific maturation of oral motor function and development has been seen as early as **15 weeks’ gestation**

- Utilizing ultrasound assessment of oral-upper airway regions in 85 fetuses, investigators concluded that oral-motor and upper airway skills emerged earlier in **females**

Miller et al., Dev Med Child Neurol 2006
Tools to Improve Oral Feeding

**NTrainer:** Somatosensory training—without feeding

Pacifier Activated Music Player (or Mother’s Voice)

**NFant:** Oromotor assessment with feeding
Current Cue Based Feeding Assessment

Infant ≥ 32 weeks’ PCA with stable respiratory status, tolerating full enteral nutrition

- Yes
  - Infant shows readiness ‘cues’
    - Yes
      - Allow to orally feed once per shift
    - No
      - Wait until ≥ 33 weeks’ PCA and reassess

- No
  - Assess ≥ 33 weeks’ PCA

Ludwig and Waitzman, Newborn and Infant Nursing Reviews 2007.
Limitations

• No ability to **assess overall developmental status of an infant in real time**
  – Why can’t a baby feed?

• No insight into developmental windows regarding **when** an intervention may prove most effective
  – Who needs what intervention when?
Reviewed the effectiveness of oral feeding assessment tools:

- Reducing length of stay
- Shortening time to establish full oral feeds

Results: “No studies met the inclusion criteria”

Conclusion: “There is currently no evidence to inform clinical practice” and research is needed in this area to develop an instrument to assess feeding readiness in the preterm infant population
Innovation

• Develop a multidisciplinary approach to address this significant knowledge gap

• Integrate molecular diagnostics with innovative tools to improve feeding outcomes in the premature neonate

NTrainer Somatosensory Integrative Machine (Barlow)

Salivary Analysis (Maron)
Multidisciplinary Team Science Approach

- **Oromotor Training**
  - Steven Barlow PhD
    - Neuroscientist and Inventor
    - University of Nebraska
    - Lincoln, NE

- **Molecular Diagnostics**
  - Jill Maron MD, MPH
    - Neonatologist and Translational Scientist
    - Tufts Medical Center
    - Boston, MA
• FDA approved device developed by Dr. Barlow improves feeding development for premature and newborn infants by reinforcing a key pre-feeding skill known as non-nutritive suck (NNS).

• Provides both assessment and therapy for diagnosing and improving a key pre-feeding skill known as non-nutritive suck (NNS).
NTrainer

Utilizes pulsatile somatosensory modulate to ‘train’ the infant how to safely suck-swallow-breathe

Family friendly, engaging of parents or caregivers

Treatment occurs during a nasogastric feed

Safe to use while on respiratory support
Progression of a Mature Suck Pattern

Initial NNS assessment

NNS assessment after starting NTrainer therapy

Final NNS assessment after NTrainer therapy

Unorganized NNS

Improved NNS

Fully organized NNS
Development of Oral Feeding Assay

• For nearly 15 years, my research has focused on the development of salivary diagnostic assays for the newborn

• Goal is to develop diagnostic assays to:
  
  1.) **Assess** an infant’s readiness to orally feed
  
  2.) **Identify** developmental delays limiting oral feeding success
  
  3.) **Personalize** our approach to treatment strategies based upon an individual’s salivary profile
Transcriptomics of Oral Feeding

• Perform real-time gene expression (RNA) profiles based upon an infant’s feeding status:
  – Successful v. Unsuccessful oral feeding

• Used various platforms:
  – RT-qPCR, microarrays and RNASeq

• Aim to gain an understanding of the developmental status of a newborn in the moment

• Conducted this research on saliva samples
Saliva as a Diagnostic Biofluid

- Saliva has several benefits over other bodily fluids
  - Noninvasive and relatively easy to obtain
  - Safe acquisition and biohazard profile
  - Parent friendly

- Direct filtrate of blood
  - Electrolytes and cells
  - Proteins, hormones, enzymes, drugs and immunoglobulins
  - Microorganisms
  - Genetic material-DNA and RNA
This research led to the need to identify a diverse gene panel for the prediction of oral feeding readiness in the premature newborn.

- Genes are representative of a diverse range of biological functions required for successful oral feeding.
- Coined Neonatal Oral-feeding Readiness In Salivary High-throughput Diagnostics (N OuRISH)

Hunger Signaling
*NPY2R, AMPK*

Sensory Integration
*PLXNA1, NPHP4*

Facial Development
*WNT3*

Maron et al. J Pediatr 2015
Positive Gene Expression

**AMPK:**
- Regulates whole body energy balance
- Activation of gene in the hypothalamus induces **feeding and weight gain**

**PLXNA1:**
- Controls axon guidance
- Increased expression in mature compared to developing **olfactory sensory neurons**

Negative Gene Expression

- **NPY2R:**
  - Down-regulated expression of this gene induces **hyperphagia**

- **WNT3:**
  - Embryologic gene involved in lip, palate and tooth formation

- **NPHP4:**
  - Involved in retinal development and visual behavior
## Successful Feeders

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive Predictive Value</th>
<th>Negative Predictive Value</th>
<th>Odds Ratio</th>
<th>Odds Ratio 95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLXNA1</td>
<td>85.05</td>
<td>22.75</td>
<td>56.12</td>
<td>56.72</td>
<td>2.89</td>
<td>(1.47, 5.67)</td>
<td>0.002</td>
</tr>
<tr>
<td>AMPK</td>
<td>96.36</td>
<td>8.38</td>
<td>55</td>
<td>66.67</td>
<td>3.21</td>
<td>(1.09, 9.48)</td>
<td>0.03</td>
</tr>
<tr>
<td>WNT3</td>
<td>17.01</td>
<td>72.46</td>
<td>41.77</td>
<td>42.91</td>
<td>0.59</td>
<td>(0.33, 1.07)</td>
<td>0.09</td>
</tr>
<tr>
<td>NPY2R</td>
<td>39.18</td>
<td>52.69</td>
<td>49.03</td>
<td>42.72</td>
<td>0.71</td>
<td>(0.36, 1.0)</td>
<td>0.05</td>
</tr>
<tr>
<td>NPHP4</td>
<td>58.25</td>
<td>35.33</td>
<td>51.13</td>
<td>42.14</td>
<td>0.60</td>
<td>(0.34, 1.03)</td>
<td>0.06</td>
</tr>
<tr>
<td>Age</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.43</td>
<td>(1.25, 1.63)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex (Female)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.75</td>
<td>(0.99, 3.06)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Results

• Data suggest again that there is no single ‘magic bullet’ biomarker for determining readiness to orally feed in the newborn

• How predictive are the biomarkers in combination?
  – Combine the 5 genes
  – Randomly select samples from the data set to generate a ROC curve
Results

- The combined expression profile of these genes, along with an infant’s post-conceptional age and sex, demonstrated 78% accuracy in predicting feeding maturity.

AUROC = 0.78

Maron et al., J Pediatr 2015.
Predictive modeling of successful oral feeders based upon age, sex and gene expression profiles

• Utilize the NOuRISH Platform to better understand
  – **Response** to treatment with the NTrainer at a molecular level
    • *Gene ontogeny over time with therapy*
  – Key developmental windows of when to **maximize NTrainer therapy**—targeting extremely premature infants
  – Identify infants who would **respond best** to Ntrainer therapy to personalize care

• Generate data regarding **both** feeding dynamic response to treatment and salivary gene expression with therapy
  – Look at data separately and together
    • *Suck dynamics + salivary gene expression*
Specific Aims

- Test the hypothesis that PULSED NTrainer stimulation of EPIs will modulate the gene expression profile of salivary feeding-readiness biomarkers and shorten duration to full oral feeds
  - Sub-Aim: Stratify infants based upon their development of bronchopulmonary dysplasia (BPD)

- Test the hypothesis that salivary feeding-readiness gene expression profiles will predict positive responders to NTrainer and an optimal neurodevelopmental stage for intervention

- Test the hypothesis that PULSED NTrainer stimulation in the neonatal period will improve feeding, growth, and neurodevelopmental outcomes at 18 months
Randomized Control Clinical Trial

- In year 5 of this multi-center clinical trial in a cohort of infants born < 29 weeks’ gestation
  - Tufts Children’s Hospital Boston, MA
  - University of Nebraska, Lincoln, NE
  - Children’s Hospital Orange County (CHOC), Orange, CA
  - Santa Clara Hospital, San Jose, CA

- One of the first neonatal salivary diagnostic clinical trials
RCT

- Infants are randomized to receive sensorimotor stimulation with the NTrainer Feeding Device or Assessment (Sham)

- Saliva samples collected throughout treatment and the learning process of oral feeding
RCT Design

**Intervention and Outcome Variables**

180 Extremely Preterm Infants (EPI) stratified by *Gestational Age*

- 24 0/7 - 26 6/7 wks
- 27 0/7 - 28 6/7 wks

Overall, ~35% are expected to develop BPD, with the remainder designated non-BPD

**Primary Outcome Variables:**
- Salivary gene expression
- Time to transition to full oral feed
- Oromotor NNS pattern formation

**Secondary Outcome Variables:**
- NICHD NRN feed-growth questionnaire @ 18 mos CA
- Bayley III

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**Schematic Key**

- **PULSED NTrainer:** 2x3 mins, 3/day
- **PULSED NTrainer:** 3x3 mins, 3/day
- **SHAM pacifier** (Std NICU practice)
- **Start nipple feed:** as early as 32 wks
- **PMA & end @ full nipple feed**
- **Start @30 wks PMA, end @D/C**

**Full oral feeds**

**Salivary Sampling Protocol:** 2 salivary samples/wk

**NNS dynamics:** 3 assessments/wk
Enrollment

• 110 infants enrolled to date (target: 140)
  – 57 males; 53 females
  – 22 infants born between 24 0/7-25 6/7
  – 88 infants born between 26 0/7-28 6/7 weeks

• 27 (25%) infants have been diagnosed with BPD

• 46 (42%) infants have completed 18-24 month follow-up
## Race and Ethnicity

<table>
<thead>
<tr>
<th>Race</th>
<th>Percent of Enrolled</th>
</tr>
</thead>
<tbody>
<tr>
<td>White</td>
<td>64%</td>
</tr>
<tr>
<td>Black</td>
<td>9%</td>
</tr>
<tr>
<td>Asian</td>
<td>8%</td>
</tr>
<tr>
<td>American Indian or Alaskan Native</td>
<td>0%</td>
</tr>
<tr>
<td>Native Hawaiian or Other Pacific Islander</td>
<td>0%</td>
</tr>
<tr>
<td>Unknown</td>
<td>15%</td>
</tr>
<tr>
<td>Multiple</td>
<td>4%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hispanic</td>
<td>51%</td>
</tr>
<tr>
<td>Non-Hispanic</td>
<td>40%</td>
</tr>
<tr>
<td>Unknown/Unspecified</td>
<td>9%</td>
</tr>
</tbody>
</table>
77 subjects (36 females, 41 males) were stratified among the two gestational age groups.

Infants randomized to receive PULSED NTrainer stimulus are treated 3x/day x 4 weeks:
- Therapy is divided into 2 phases, in 2-week block intervals:
  - Phase 1: 3 min training x 2; 1 min pause
  - Phase 2: 3 min training x 3; 1 min pause

Subjects randomized to the SHAM condition were given a regular silicone pacifier during tube feedings over the same time period, and same developmental care as those infants in the experimental group of the study.
Advancing Analytics: NeoNNS.exe

NNS raw data

Raw NNS input pressure

Auto NNS burst selection
- Demean pressure waveform
- Suck peak picker
- NNS burst discriminator

Statistical Analysis

- Linear mixed modeling (LMM) analysis was conducted for each dependent variable (NNS dynamics) to examine differences:
  - Between patient type (BPD, RDS)
  - Treatment type (NTrainer, Sham),
  - Therapy phase as well as interactions among those three factors

- The models accounted for patients’ age (GA, PMA) and sex and dependency of observations repeated within patients (i.e., intraclass correlation), thereby providing unbiased estimates of the model
Predicting Feeding Based on NNS Dynamics

Figure 4. (a) Tsfresh cluster heat map based on 568 NNS files from 30 EPI babies after feature elimination (p<1.35e-22). Red is positive, green is negative and blue is unknown. The x-axis represents Tsfresh features and the y-axis represents NNS assessment file records. The corresponding indexes mapping Tsfresh features (x-axis) and NNS files (y-axis) are saved in these complementary files, “mapping_heatmap_features.xlsx” and “mapping_heatmap_nns.xlsx”. (b) NeoNNS cluster heat map of all NNS files based on 11 NeoNNS features (p<1.70e-23). All the parameters are the same as used in the Tsfresh cluster heat map (Liao et al., 2019).
Observation

- One of the early observations of the trial was how well the male infants seemed to respond to therapy
  - Achieve oral feeds earlier than female infants if given the NTrainer

- This observation was purely anecdotal, but consistent across all sites

One size does not fit all.
• There still remains a knowledge gap regarding the essential molecular mechanisms required for oral feeding maturation
  – Particularly based on sex

• We hypothesized that the RNA Seq platform would:
  – *Improve our understanding of oral feeding competency*
  – *Identify novel pathways related to oral feeding success not previously seen*
  – *Personalize treatment strategies*
Methods

- Performed RNASeq on saliva samples collected from both successful and unsuccessful oral feeders

- Cohorts were matched by gestational age, post-conceptional age, sex, and ethnicity

- Performed comparative and systems biology analyses of differentially expressed genes between
  - Successful and unsuccessful oral feeders
  - Males and females

Maron et al., 2021 in prep
Results

- Overall, 63 genes were differentially expressed between feeders and non-feeders
  - 59 mapped to a known gene function; 4 genes were unmapped
• Analysis highlighted other areas of biological relevance including disruption in:
  
  – Palatal shelf formation
  
  – Maturation of circadian rhythms
  
  – Abnormal morphology of hindgut and mesenchyme
  
  – Development of the abdomen
Independent analyses of males and females highlighted the unique differences in oral feeding maturation between the sexes.

88 genes (♀)  
77 genes (♂)  

NO OVERLAP

Maron et al., 2021 in prep
Males

• 77 genes were differentially expressed between feeders and non-feeders
  • 72 mapped to a known gene function; 5 genes were unmapped
Females

- 88 genes were differentially expressed between feeders and non-feeders
  - 85 mapped to a known gene function; 3 genes were unmapped
Disrupted Developmental Pathways

Nervous System Development and Function
p values: < 0.04 to < 0.0008
n = 6 genes

Hair and Skin Development
p values: < 0.05 to < 0.003
n = 4 genes

Cardiovascular System Development and Function
p values: < 0.05 to < 0.003
n = 7 genes

Connective Tissue Development and Function
p values: < 0.05 to < 0.003
n = 8 genes

Embryonic Development
p values: < 0.05 to < 0.003
n = 6 genes
Disruption in memory and learning was only seen in male subjects

- Abnormal morphology of hippocampal CA1 regions
  - CA1 is required for contextual memory retrieval
  - Re-experiencing detailed episodic memories

Facial, palate and gastrointestinal development was driving significance in gene expression in female subjects
Expanded NOuRISH Platform

- Incorporating additional genes onto the NOuRISH Platform based on subsequent analysis on the RNA-Seq platform

- Genes related to hippocampal development and memory will now be included

- Hypothesize that expression of these genes may discern between babies that would benefit from the NTrainer and those who require a different therapy
• Over 2,000 saliva samples have been collected from the 110 subjects enrolled to date

• Samples will be analyzed on the amended NOuRISH platform this spring

• 18-24 month neurodevelopmental follow-up visits with feeding questionnaires are being conducted nationwide
  – Understand the long-term impact of the study
Oliva Koumantzelis and her mother Sarah
Acknowledgments

• Families who graciously participated

• Nurses and Staff in the NICU at Tufts Medical Center, University of Nebraska, Children’s Hospital of California, and Santa Clara Hospital

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