Novel Approaches to Oral Feeding Readiness Assessment in the Newborn

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Disclosures

- I have no financial disclosures or conflicts of interest.
Objectives

- To recognize the complex and diverse biological mechanisms involved in oral feeding in the newborn

- To recognize the benefits of using saliva as a diagnostic biofluid in the newborn

- To recognize the need for improved objective assessment tools for neonatal oral feeding abilities
Clinical Dilemma: Oral Feeding

- **Majority** of 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 to AAP guidelines.
The most complex neurological task of the newborn is the ability to successfully orally feed.

To effectively feed while protecting the airway an infant must integrate:
- Nervous system
- Musculoskeletal system
- Gastrointestinal system
- Respiratory system
- Sensory systems (vision, touch, smell, hearing)
Oral Feeding Readiness

- Current standard of care depends upon subjective assessment to determine oral feeding readiness in the newborn:
  - Nursing
  - Physical, occupational and speech therapists
  - Cue-based feeding algorithms
Infant $\geq 32$ weeks’ PCA with stable respiratory status, tolerating full enteral nutrition

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

- No
  - Assess $\geq 33$ weeks’ PCA

Reviewed the effectiveness of oral feeding assessment tools in:

- 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

Failed feeding attempts

- Feeding babies we shouldn’t and not feeding babies we could
- Estimated 40% of children in feeding disorder clinics are former preterm infants

Prolonged length of stay

- Ability to successfully feed by mouth is one of the major determinants for length of stay in the NICU

Millions of health care dollars annually

- Reducing length of stay may translate into millions of dollars in health care costs savings

• Failure to develop normal feeding patterns by term gestation correlates with impaired neurodevelopment
  – *Significantly lower mental and physical developmental scores at 6 and 12 months*
  – *Impaired neurodevelopmental outcomes at 18 months*

• Thus, oral feeding at term gestation serves as the first neurological assessment of the newborn

Anecdotes About Feeding

• Various opinions about why newborns can’t orally feed
  – Babies with IDDM are ‘pokey’
  – Babies with PPHN have been ‘sick’
  – Babies with NAS are not ‘captured’
  – 35 weeker who fed well earlier in day is now ‘too tired’

• Uniform treatment strategies
  – Give it time
  – Consult OT/PT +/- speech therapy
  – May consult surgery for a gastrostomy tube
Oral Feeding

• Why don’t we have an **objective** oral feeding assessment assay?

• **Babies do not feed for different reasons**

• We need to be able to objectively monitor multiple developmental systems **simultaneously**
  – Oral motor control and facial development
  – Sensory integration (olfactory, vision, hearing, taste)
  – Hunger signaling
  – Neurodevelopment
  – Gastrointestinal development
My laboratory hypothesized that neonatal salivary gene expression analysis would provide novel, comprehensive and objective evidence about an infant’s readiness to orally feed.
Why Saliva?

• Saliva has several benefits over other bodily fluids
  – Noninvasive and relatively easy to obtain
  – Safe acquisition and biohazard profile

• Direct filtrate of blood
  – Electrolytes and cells
  – Proteins, hormones, enzymes, drugs and immunoglobulins
  – Microorganisms
  – Genetic material: DNA and RNA
Analysis of neonatal saliva is not novel

- Proteins — ie, cortisol levels and stress response
- Microorganisms — ie, neonatal salivary CMV studies

It was novel to study saliva gene expression

- Historically, the inherent instability of single-stranded RNA had made gene expression impossible to analyze in saliva
- ~2005, commercial assays became available that allowed for the stabilization and subsequent analysis of RNA (gene) targets
First, we had to address technical considerations

1.) **Can we do it?**

   Assays were not developed for neonates

2.) **Would it be informative?**

   Could we gain a better understanding of a infant’s ability to feed through salivary gene expression analysis?
Saliva Collection

1 mL syringe

Wall suction

20 seconds

Ice immediately

To laboratory

Salivary sample (10 µL)

Samples are stable at 4°C for up to 4 weeks before processing

Birth weight: 500 gm to ≥4000 gm

Images courtesy Jill L. Maron, MD, MPH

• Proof of principle study to assess the benefits of neonatal salivary gene expression analysis
• Recruited premature infants born between 28 to 32 weeks’ gestation
• Collected saliva throughout an infant’s hospitalization

Specifically around feeding milestones—enteral advancement, oral feeding

Initial Salivary Study

Multiple Samples Collected

Birth 28 30 32 34 36 38 Post-Conceptional Age (weeks) Discharge
Initial Salivary Study

• Performed comparative gene expression microarray analyses of samples collected over time

• 5 feeding stages
  1. No enteral nutrition (NPO)
  2. Partial per gastric feeds (PPG)
  3. Full gastric feeds (FPG)
  4. Partial oral feeds (PPO)
  5. Full oral feeds (FPO)

• Each infant served as his/her own control
Analysis

Gene expression assays → Bioinformatic analysis

Systems biology-IPA®

Images courtesy Jill L. Maron, MD, MPH
Analysis

- IPA® identifies gene-gene relations, associated network functions, and physiological developmental systems

- IPA® determines the probability that the association between genes present in a given list and a given biological process was due to random chance

- For a targeted analysis of the data, we only considered those genes that met statistical criteria and were associated with the keywords “feeding”, “digestion” and “development”
There were 2,186 genes that met criteria and appeared to be related to feeding:

379 genes (18%)

1,807 genes (82%)

We were able to simultaneously detect genes involved in:

- Innervation of Oral Muscles (Cranial Nerves)
- Sensory Input (Smell, Vision, Hearing)
- Neurodevelopment
- GI Development (Motility)
- Feeding Behavior

Feeding Behavior

- Identification of feeding behavior pathways in newborns learning to feed is novel

- Limited molecular data are available:
  - Hunger signaling
  - Satiety
  - Neuronal regulation of food intake
  - Hypothalamic regulation of feeding behavior

- The important role of biomarkers involved in feeding behavior makes biological sense in the newborn
On average, a newborn infant gains **200%** of his/her birth weight by 1 year of age

- *A preterm infant may gain >300% of his/her birth weight*

- Newborn must consume 80-150 kcals/kg/day

- Caloric intake of a newborn is equivalent to an adult diet of 7,000 to 10,000 kcals/day
Hypothalamus and Neonatal Feeding

- Infants must demonstrate exponential weight gain postnatally
- We hypothesized that hypothalamic maturation is necessary for successful oral feeding in the premature newborn

Photo: Sean O’Riordan
Photo: Victor Shapiro
Gene of Interest: *NPY2R*

- Neuropeptide Y2 receptor (*NPY2R*) was a gene identified in ‘feeding behavior’ in IPA®

- Known to be associated with feeding behavior, metabolism, and energy homeostasis

- Known to be dysregulated in patients with obesity
  - *When the gene is down-regulated, individuals overeat*

- Target of novel drug therapy for the treatment of obesity

- It is predominantly expressed in the arcuate nucleus of hypothalamus
  - *Permeable to the blood brain barrier rendering it detectable in saliva*
In 1999, Naveilhan and colleagues were the first to generate a knock-out mouse model for NPY2R.

Gene of Interest: **NPY2R**

Weighed 180% more than controls

♀ > ♂

Images courtesy Jill L. Maron, MD, MPH

We hypothesized that when infants were ready to orally feed, they would **down-regulate** *NPY2R* gene expression.

Developed a RT-qPCR assay for *NPY2R* and tested it on healthy-term neonatal samples.

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Neonatal *NPY2R* Expression

- **Biobank Samples**
  - Preterm neonates at various postconceptional ages and feeding stages
  - Healthy term neonates
  - 116 salivary samples from 76 infants

- **Performed RT-qPCR for *NPY2R* in salivary samples:**
  - Multiplex one-step assay on extracted salivary total RNA
  - Each sample was analyzed for 3 reference genes + *NPY2R*
  - *NPY2R* was run in triplicate with each reference gene

- **Expression of *NPY2R* was correlated with PCA and feeding status**

NPY2R as a Biomarker

- NPY2R performed in a binary fashion
  - Likely a consequence of the detection level on the RT-qPCR platform in neonatal saliva

- Amplification of NPY2R in neonatal saliva has a 95% positive predictive value in determining that an infant cannot sustain full oral feeds

- However, the negative predictive value of the assay was only 27%

NPY2R as a Biomarker

- NPY2R is a highly promising salivary biomarker

- Research suggests that the maturation of the hypothalamus plays an important role in successful oral feeding in the newborn

- However, NPY2R cannot be the only marker to determine readiness to feed

- Need to consider all aspects of oral feeding for the development of a diagnostic assay

Just a piece of the puzzle
Prospective salivary gene expression microarray analyses on a new cohort of infants (n=12)
  – Considered 2 feeding time points: partial and full oral feeds
  – Samples were collected over a short amount of time
  – Limit gene expression changes representative of other developmental processes

Two analytical approaches were used to identify potential salivary biomarkers

Candidate biomarkers were selected following a systems biology analysis.
Limitations to Systems Biology

- **Bias from the investigator**
  - Limited by the clinical acumen and prejudice of the investigator

- **Bias from the scientific literature**
  - Only can identify biomarkers based on what is published
  - Annotation bias in databases
    - *Gene function is largely defined by adult studies*

- **Limited by what is known**
  
  What about the unknown?
• Computational analysis of the microarray data
• Identify gene targets in an unbiased fashion
• Discover potentially novel genes and gene-gene relationships as they relate to oral feeding in the newborn

Dr. Gil Alterovitz
Harvard Medical School

Images courtesy Jill L. Maron, MD, MPH
NOuRISH Assay

- NOuRISH: Neonatal Oral-feeding Readiness in Salivary High-throughput Diagnostics
- Custom RT-qPCR assay composed of 24 genes, inclusive of 3 reference genes

Images courtesy Jill L. Maron, MD, MPH
• 400 salivary samples collected from 298 infants were run on the NOuRISH platform

• Salivary samples included:
  – 200 successful feeders (>32 weeks to 48 weeks’ PCA)
    • 100% of feeds by mouth
  – 200 unsuccessful feeders (>31 weeks to 44 weeks’ PCA)
    • <100% of feeds by mouth

• Samples were prospectively collected and correlated to feeding status
Genes were considered in a binary fashion
  – (+/- gene expression based upon our threshold of detection)

Statistical analyses included a multi-variable analysis to control for PCA and sex
  – OR, Sensitivity, Specificity, PPV, NPV

Fitted probability tables were generated to assess the likelihood an infant could successfully feed based upon PCA, sex and gene expression profile

Assessed an infant’s readiness to succeed

NOuRISH Results

- No statistically significant difference between infants who received breast milk in successful and unsuccessful oral feeders ($P=.07$)

- 90% of salivary samples amplified successfully
  - Defined as the amplification of the 3 reference genes: GAPDH, ACTB, YWHAZ

- 20/21 target genes successfully amplified
  - 1 gene failed to amplify in any sample
    - *Presumably in a spliced exon*

Results

- After controlling for postconceptional age and sex, 5 genes were further considered on the platform.

- Hunger Signaling: NPY2R, AMPK
- Sensory Integration: PLXNA1, NPHP4
- Facial Development: WNT3

Images courtesy Jill L. Maron, MD, MPH

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>Factors</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>.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>.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>.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>.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>.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;.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>.05</td>
</tr>
</tbody>
</table>

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 an ROC curve

Results: AUROC

- In combination, these 5 markers have very good accuracy at predicting feeding success in the premature newborn.
- This approach is a significant improvement over ‘best guess’ estimates currently used in clinical practice.

AUROC = 0.78

Predictive modeling of successful oral feeders based upon age, sex and gene expression profiles

Predictive modeling of successful oral feeders based upon age, sex and gene expression profiles

Predictive modeling of successful oral feeders based upon age, sex and gene expression profiles

Limitations

- The exact biological mechanism by which each of these genes is affecting oral feeding is unclear
  - Rare for one gene to have only one function

- These were **human translational** studies
  - There was no experimentation, intervention or intention to treat
  - We neither inflicted harm nor deviated from clinical care

- We can only speculate on their role in feeding success
  - Complex computational analysis
  - Biological plausibility and gene expression patterns
Next Steps

- Further understand the biological mechanisms involved in neonatal oral feeding
  - Utilizing RNASeq to examine transcriptional regulation
  - Enhance biomarker discovery

- Improve our understanding of an infant’s ability to feed on the long-term developmental outcomes of the newborn
  - Developmental follow-up testing
  - Speech language emergence
Collaboration with Dr. Emily Zimmerman, speech pathologist at Northeastern University

Targeting potential biomarkers linking oral feeding maturation with speech language emergence

Forkhead box protein 2 (FOXP2) was the first gene to be implicated in a developmental disorder of speech and language

- Molecular studies of 15 individuals in the ‘KE’ family who suffered from speech language disorders

Prospective study correlating relative quantitative salivary *FOXP2* gene expression levels with:

- Duration of time to learn to orally feed (days)

- Infants born between 30 and 34 weeks’ GA (n=20)

- Saliva samples obtained at time of first oral feeding attempts

- Performed multiplex RT-qPCR for quantification of *FOXP2* with appropriate controls
FOXP2

<table>
<thead>
<tr>
<th>Sex</th>
<th>GA</th>
<th>Birth weight (g)</th>
<th>PCA at salivary collection</th>
<th>PCA at full oral feeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male = 13</td>
<td>32.33 (1.04)</td>
<td>1.858 (289)</td>
<td>33.44 (0.78)</td>
<td>35.03 (1.27)</td>
</tr>
<tr>
<td>Female = 7</td>
<td></td>
<td></td>
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</tbody>
</table>

*Mean +/- SD

- Performed a linear regression analysis controlling for sex and GA
- Quantitative FOXP2 gene expression levels were found to be significantly associated with a shortened duration to achieve successful oral feeds (\(P= .043\))

Zimmerman et al. in press.
“I came across your name while researching my son’s recent diagnosis.”

My son “was born via c-section at exactly 35 weeks because I had preeclampsia. He suffered no trauma during pregnancy or labor.”

“He was in the NICU for 42 days for ‘suck, swallow, breathe’ immaturity. We tried breastfeeding, formula, thickened formula, different nipple sizes, spot feeding etc.”
“We had no explanation for why he couldn't coordinate SSB. He underwent an ultrasound of both his brain and his heart, and he had an MRI. All findings were normal or nonsignificant.”

“. . . after failing a swallow test with flying colors, he had a g-tube placed . . .”

“In an effort to find the cause of the issue, his neonatologist ordered a microarray and chromosomal analysis. . . . . ~9kb loss within chromosome band 7q31.1 that contains exon 2 of FOXP2 gene”
Summary

• Oral feeding is an important, biologically complex, neurodevelopmental milestone
  – Learning to feed impacts nearly every baby in our care
  – **NOT** a one size fits all problem
  – Causation of poor oral feeding skills will likely inform us about potential risks to developmental impairment

• Need to develop objective assessment tools to assess feeding maturity and to identify disrupted developmental pathways limiting feeding success
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