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

Jill L. Maron M.D., M.P.H.
Professor of Pediatrics
February 2, 2021

Tufts Medical Center
Mother Infant Research Institute

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**

Beam AL *et al. J Perinatol* 40, 1091–1099 (2020).

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

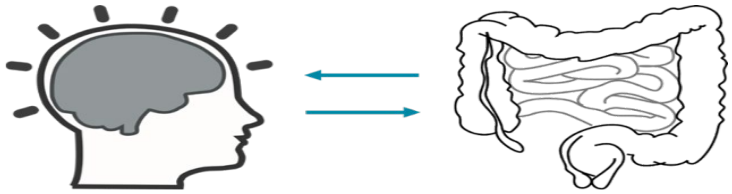
- **Oral feeding competency relies upon the maturation and coordination of:**

Suck ⟶ ***Swallow*** ⟶ ***Breathe***

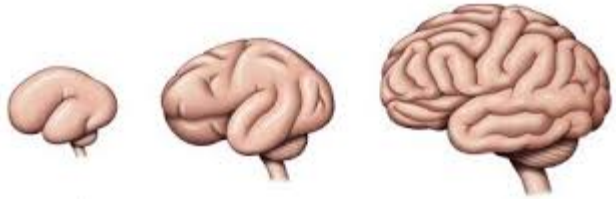
- **However, the ability to feed is also driven by:**

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

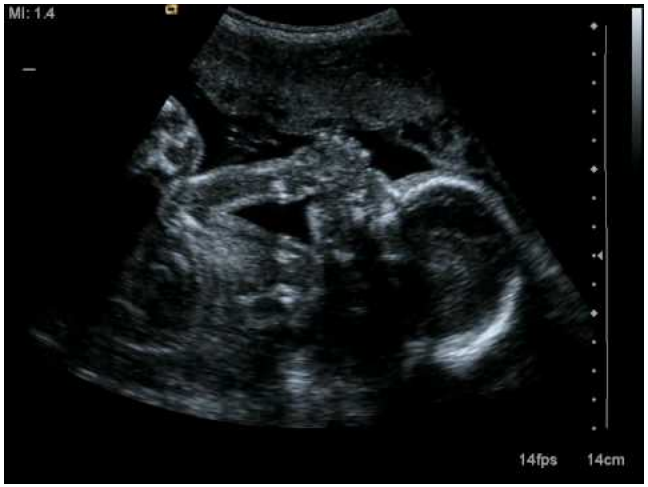
Explanatory (independent) variable	Estimate	95% Confidence limits		p-Value
PMA at first feed (in weeks)	4.49	4.13	4.85	<0.0001
Birth weight (per 100 g)	−0.46	−0.51	−0.40	<0.0001
SGA	0.91	0.24	1.58	0.008
Male	1.31	0.87	1.76	<0.0001
Surfactant exposure	2.43	1.87	3.00	<0.0001
PDA requiring treatment	3.37	0.93	5.81	0.007
Black race	−1.62	−2.44	−0.80	0.0001
Multiple gestation	0.59	0.09	1.10	0.021
Human milk in the first 28 days	0.77	0.11	1.44	0.023

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

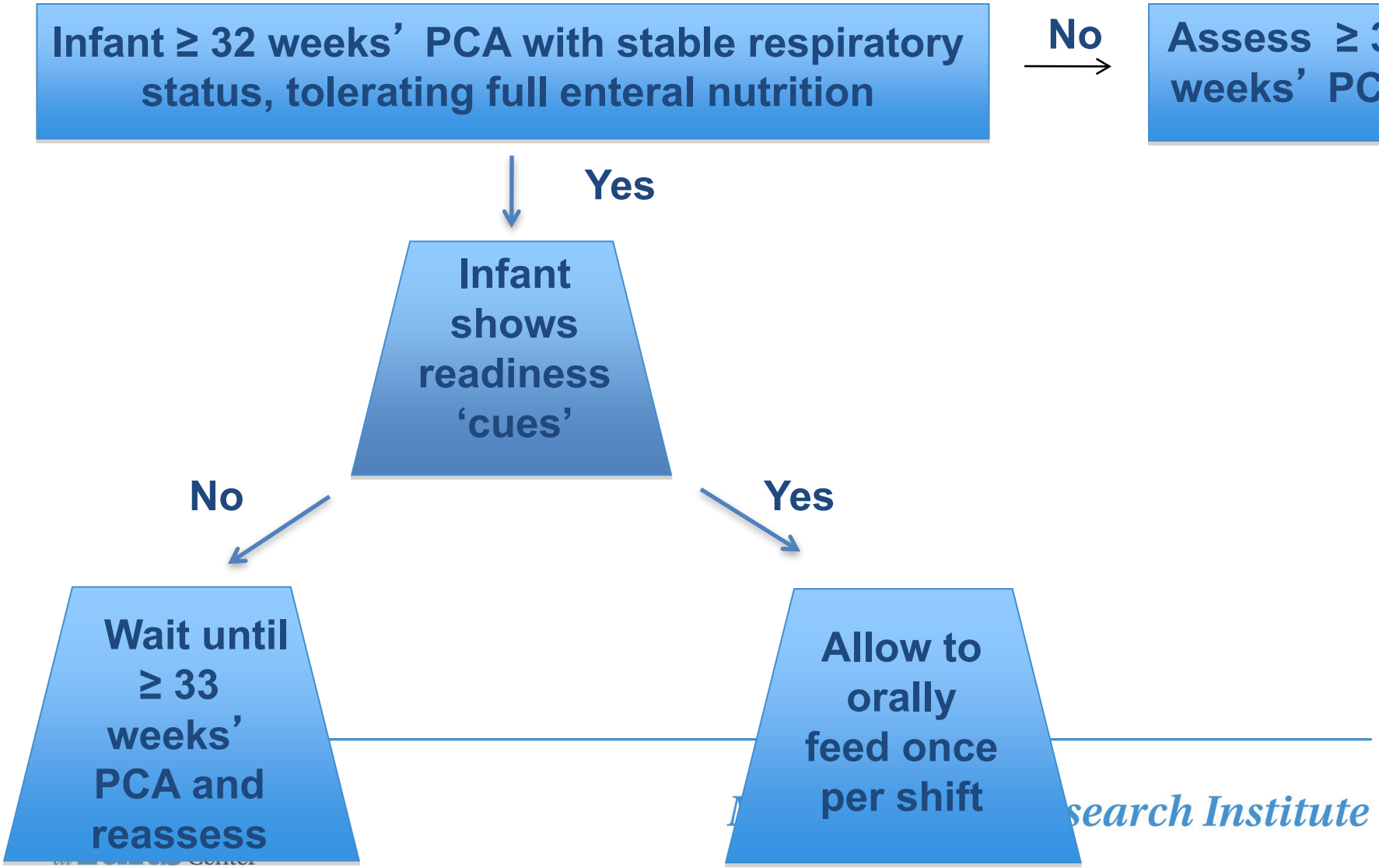
Tools to Improve Oral Feeding

NTrainer: Somotosensory training—without feeding

Pacifier Activated Music Player (or Mother's Voice)

NFant: Oromotor assessment with feeding

Current Cue Based Feeding Assessment



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?

Cochrane Review 2012 and 2016

- 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

Crow et al., Cochrane Database Syst Rev. 2012 Apr 18;4:CD005586.
Crow et al., Cochrane Database Syst Rev. 2016 Aug 23;8:CD005586.

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

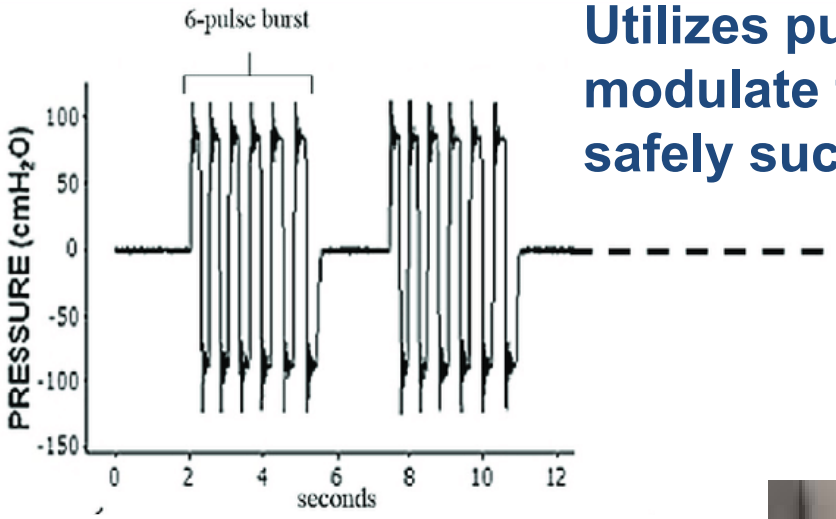
- **Molecular Diagnostics**

Jill Maron MD, MPH
Neonatologist and Translational Scientist
Tufts Medical Center
Boston, MA

NTrainer

- 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



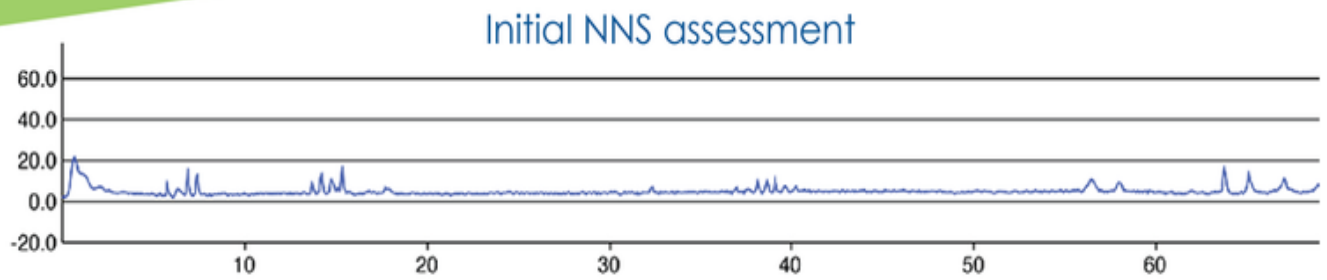
Utilizes pulsatile somatosensory modulate to 'train' the infant how to safely suck-swallow-breathe

Family friendly, engaging of parents or caregivers

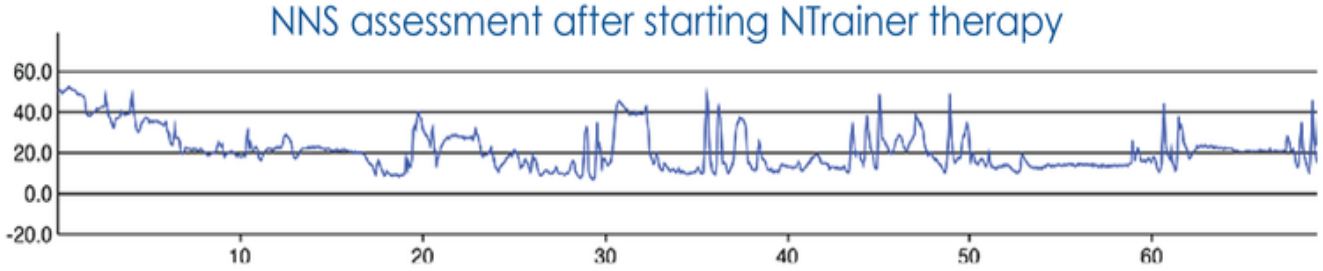
Treatment occurs during a nasogastric feed

Safe to use while on respiratory support

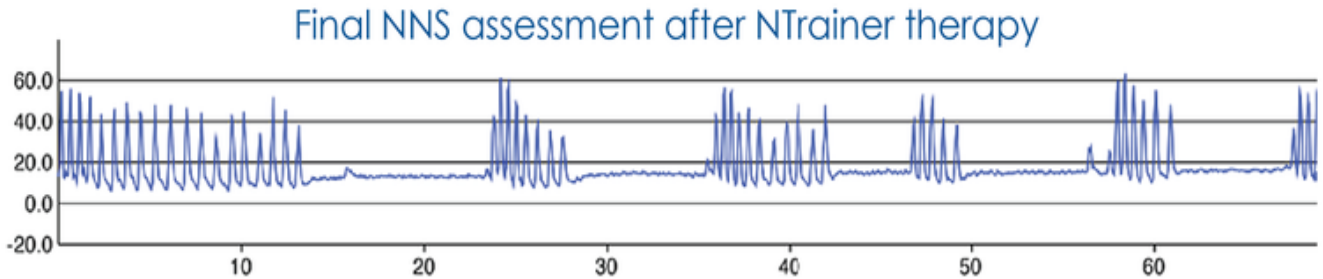
Progression of a Mature Suck Pattern



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

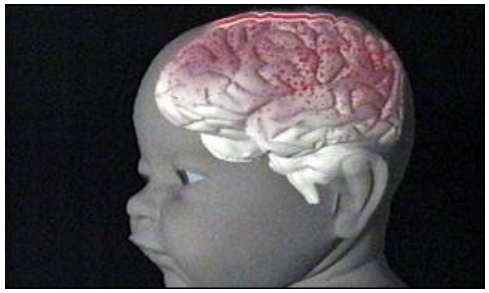
Saliva as a Diagnostic Biofluid

- **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**

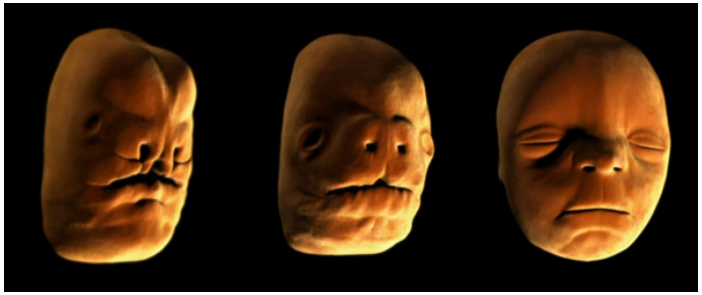
Background

- 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 (NOuRISH)



Hunger Signaling
NPY2R, AMPK

Sensory Integration
PLXNA1, NPHP4



Facial Development
WNT3

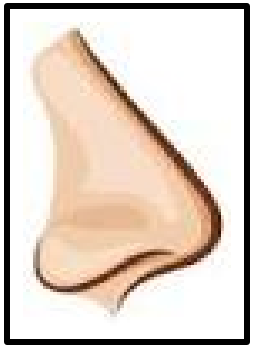
Maron *et al. J Pediatr 2015*

Positive Gene Expression

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

AMPK = Hunger

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



PLXNA1 = Olfactory maturation

McIntyre *et al.*, *J Neurosci Res* 2010 88:3243-3256.

Negative Gene Expression

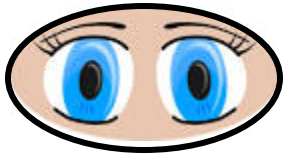
- ***NPY2R:***
  - ***Down-regulated expression of this gene induces hyperphagia***

***NPY2R* = Hunger**

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

***WNT3* = Facial Development**

- ***NPHP4:***
  - ***Involved in retinal development and visual behavior***



***NPHP4* = Vision**

Successful Feeders

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

Maron *et al., J Pediatr* 2015 166(2): 282-288.

Results

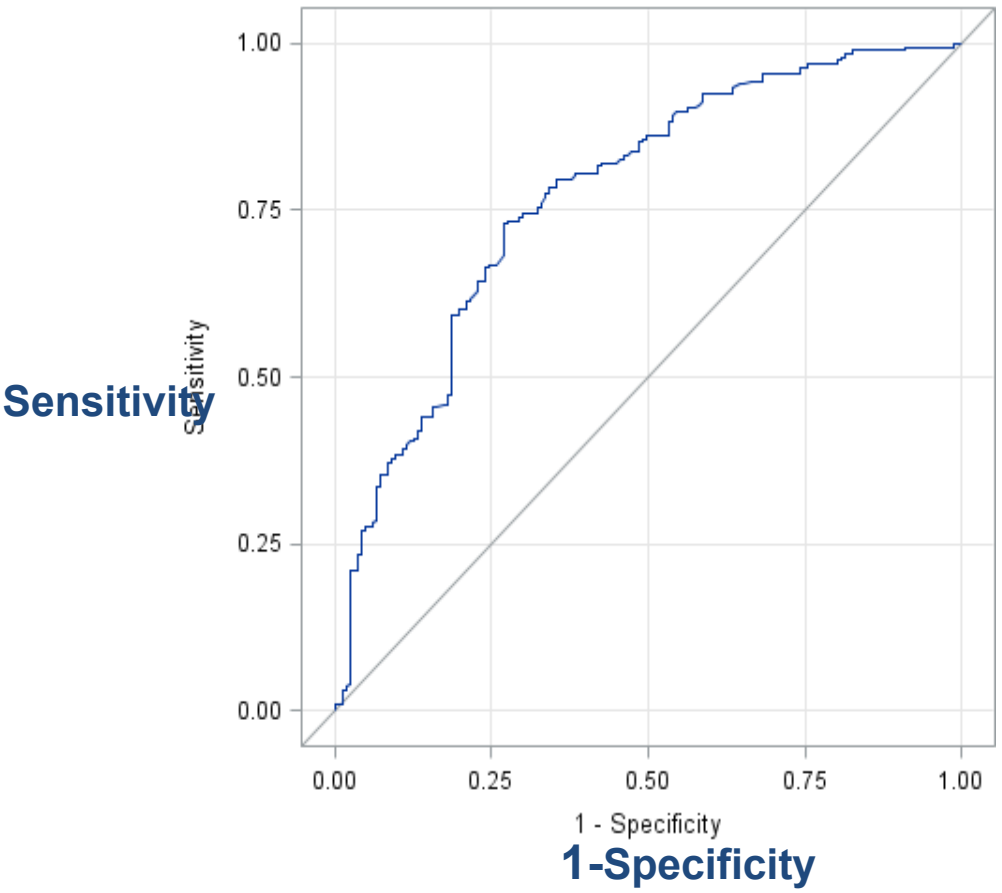
- Data suggest again that there is no single 'magic bullet' biomarker for determining readiness to orally feed in the newborn

- 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 theses 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: Feeding Success

35 week ♂

NPY2R	WNT3	NPHP4	AMPK − / PLXNA1 −	AMPK − / PLXNA1 +	AMPK + / PLXNA1 −	AMPK + / PLXNA1 +
−	−	−	14%	32%	35%	60%
−	−	+	9%	22%	34%	48%
−	+	−	9%	22%	24%	48%
−	+	+	6%	14%	16%	35%
+	−	−	9%	22%	24%	48%
+	−	+	6%	15%	16%	35%
+	+	−	6%	15%	16%	36%
+	+	+	3%	9%	10%	25%

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

Maron *et al., J Pediatr* 2015 166(2): 282-288.

Strategy

- 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**

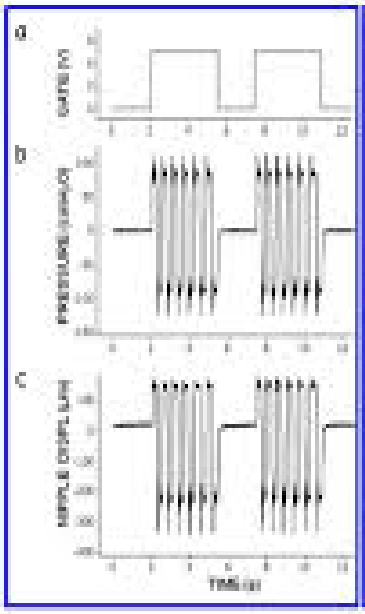
- **One of the first neonatal salivary diagnostic clinical trials**

NIH U.S. National Library of Medicine

ClinicalTrials.gov

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

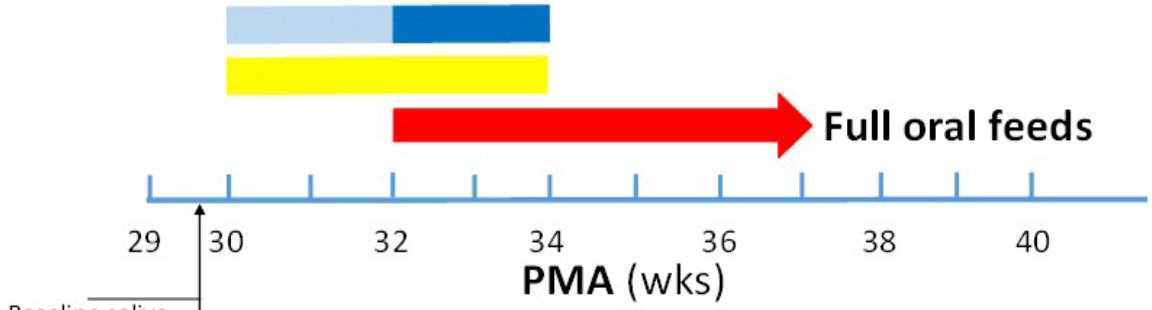
Overall, ~ 35% are expected to develop **BPD**, with the remainder designated **non-BPD**

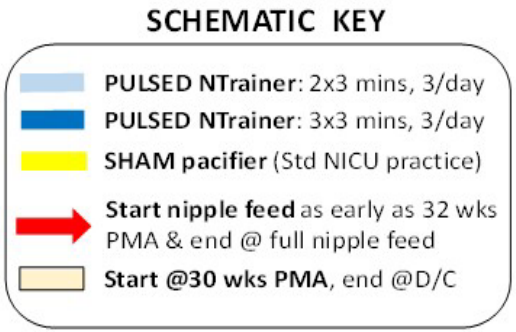
Primary Outcome Variables:

- 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





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

Race	Percent of Enrolled
White	64%
Black	9%
Asian	8%
American Indian or Alaskan Native	0%
Native Hawaiian or Other Pacific Islander	0%
Unknown	15%
Multiple	4%
Ethnicity	
Hispanic	51%
Non-Hispanic	40%
Unknown/Unspecified	9%

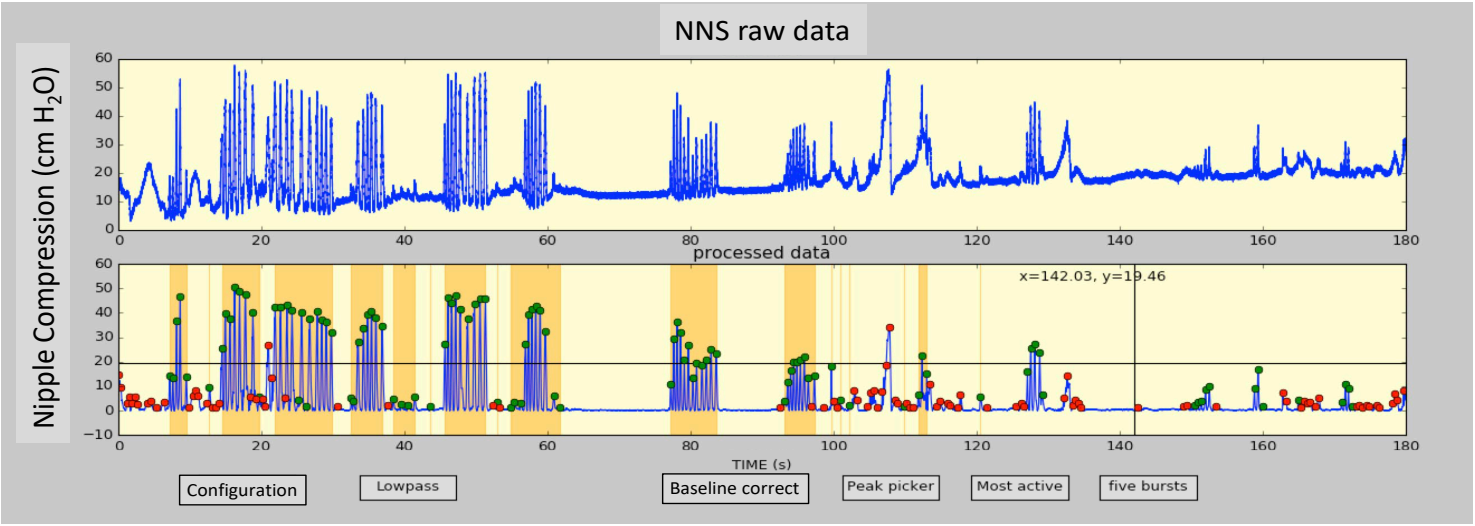
NNS Dynamics: Interim Analysis

- 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

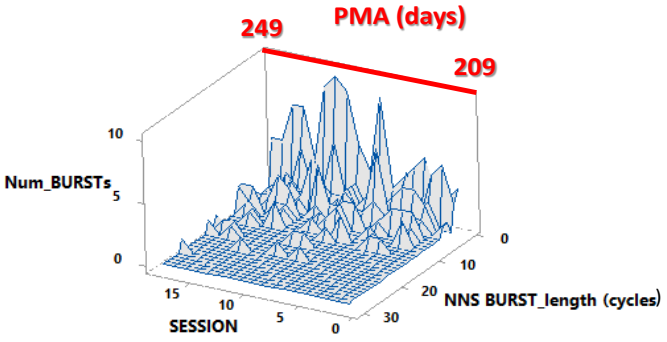
Advancing Analytics: NeoNNS.exe



Raw NNS input pressure

Auto NNS burst selection

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



- **Liao, Rosner, Maron & Barlow. (2017).** Automatic Non-nutritive Suck Waveform Discriminator and Dynamic Feature Extraction in Preterm Infants. Neonatal Neurology: Preterm Newborns, *Pediatric Academic Society*, San Francisco, CA. **3853.12: 555**.
- **Liao C, Rosner AO, Maron JL, Song D, Barlow SM. (2019).** Automatic non-nutritive suck waveform discrimination and feature extraction in preterm infants. *Computational and Mathematical Methods in Medicine*, 2019, 7496591, https://doi.org/10.1155/2019/7496591.

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

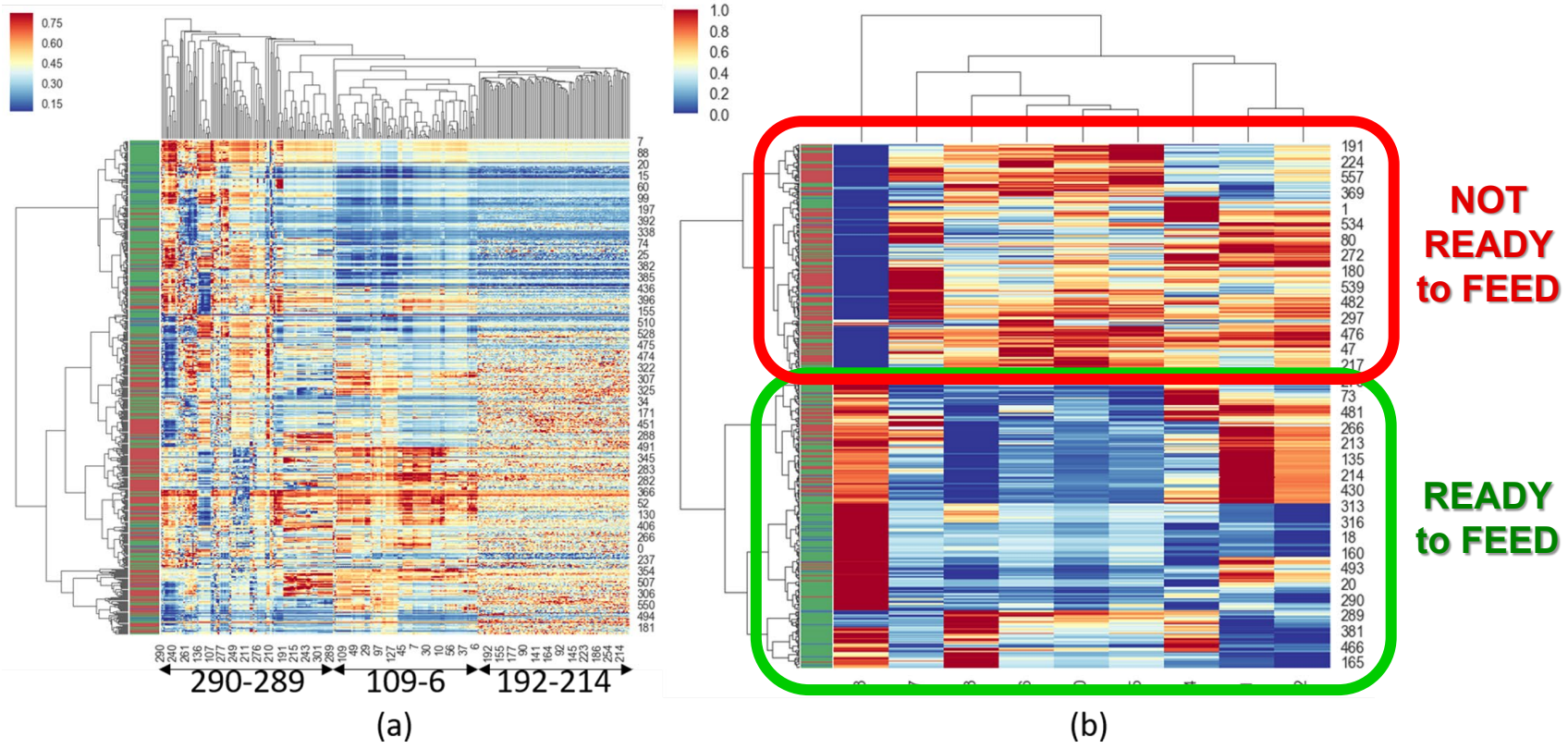
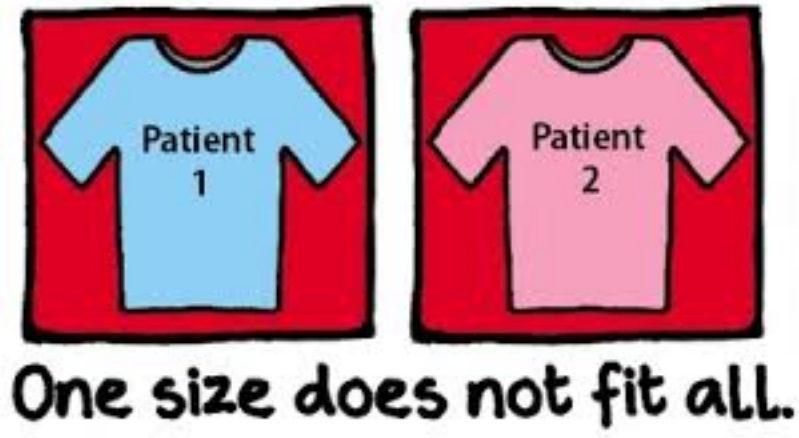


Figure 4. (a) Tsfresh cluster heat map based on **568 NNS files from 30 EPI babies** after feature elimination (p<1.35e-22). **Red** is positive, **green** is negative and **blue** is unknown. The x-axis represents Tsfresh features and the y-axis represents NNS assessment file records. The corresponding 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 the NTrainer

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



Needed Additional Studies

- **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***

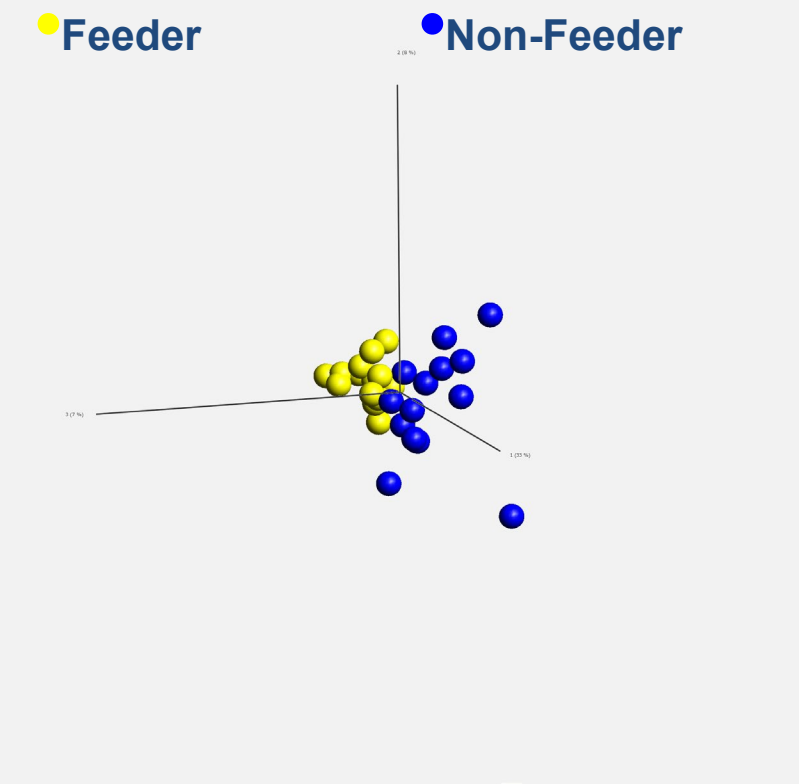
Methods

- 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

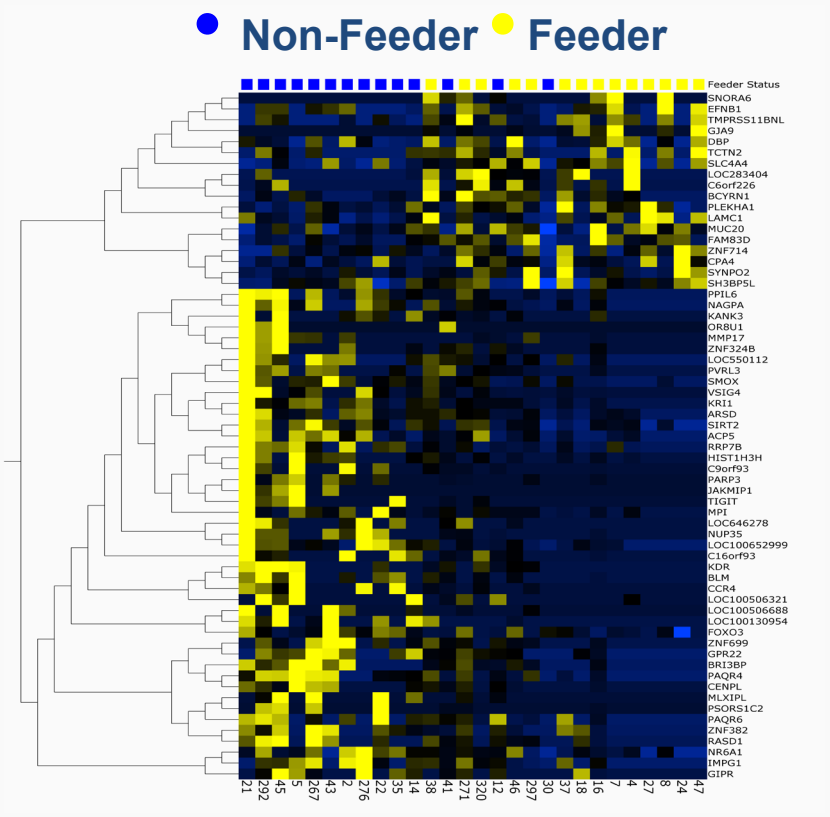
Maron et al., 2021 *in prep*

Results

- **Overall, 63 genes were differentially expressed between feeders and non-feeders**
  - ***59 mapped to a known gene function; 4 genes were unmapped***



PCA Plot



Heat Map

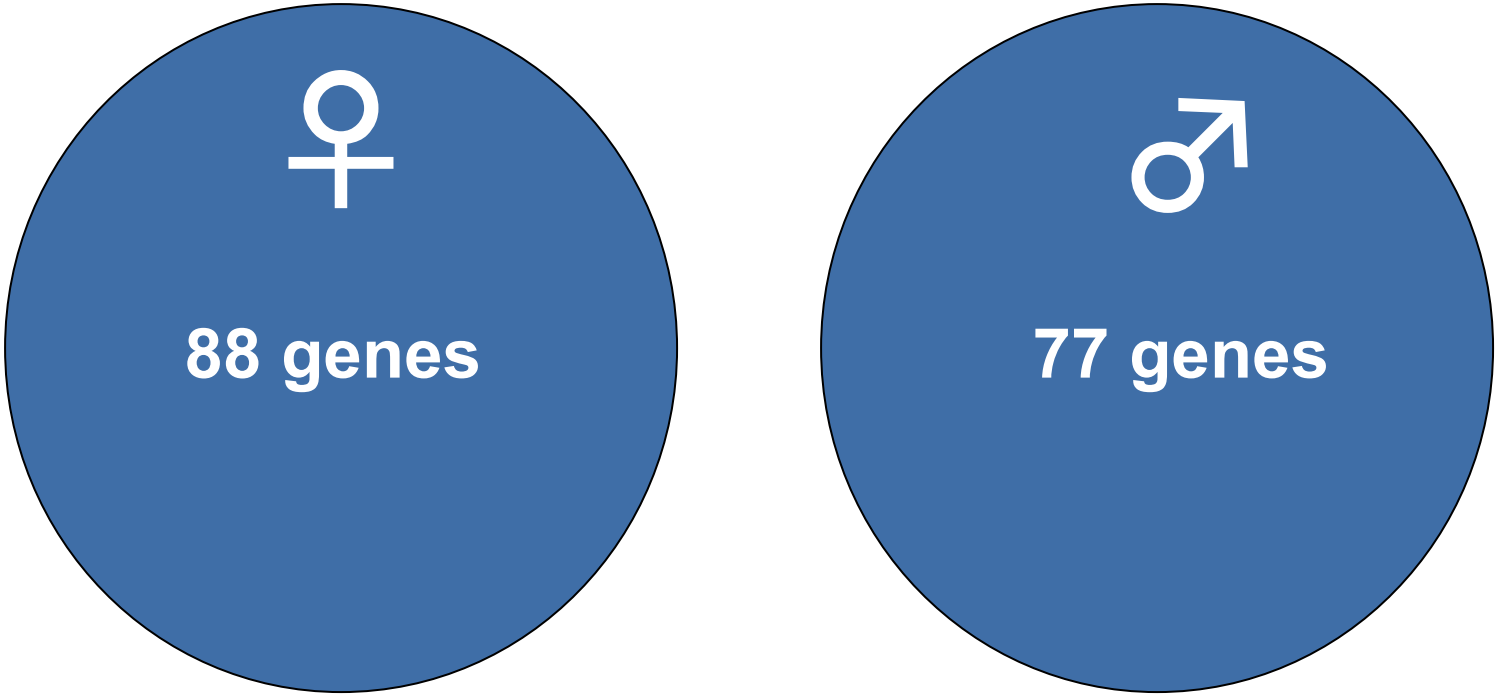
Networks of Interest

- **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**

Maron et al., 2021 *in prep*

Sex Matters

Independent analyses of males and females highlighted the unique differences in oral feeding maturation between the sexes

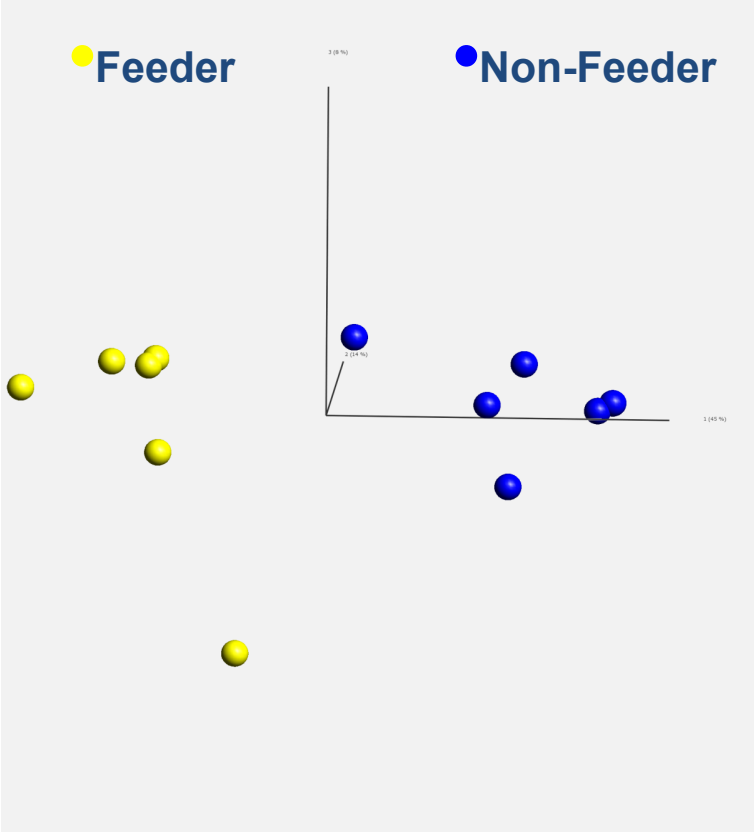


NO OVERLAP

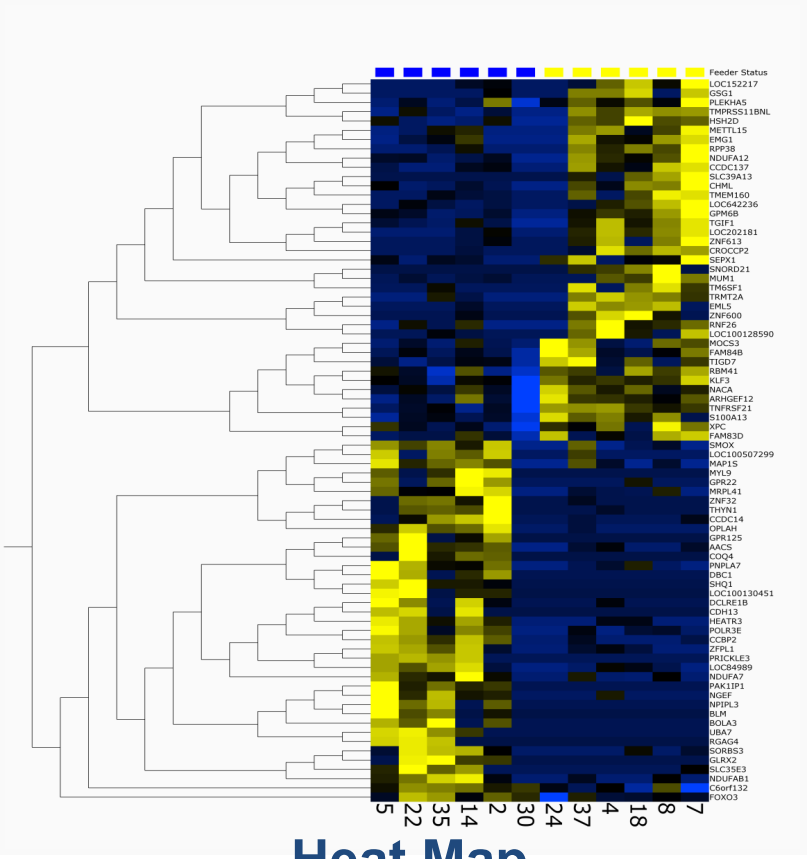
Maron et al., 2021 *in prep*

Males

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



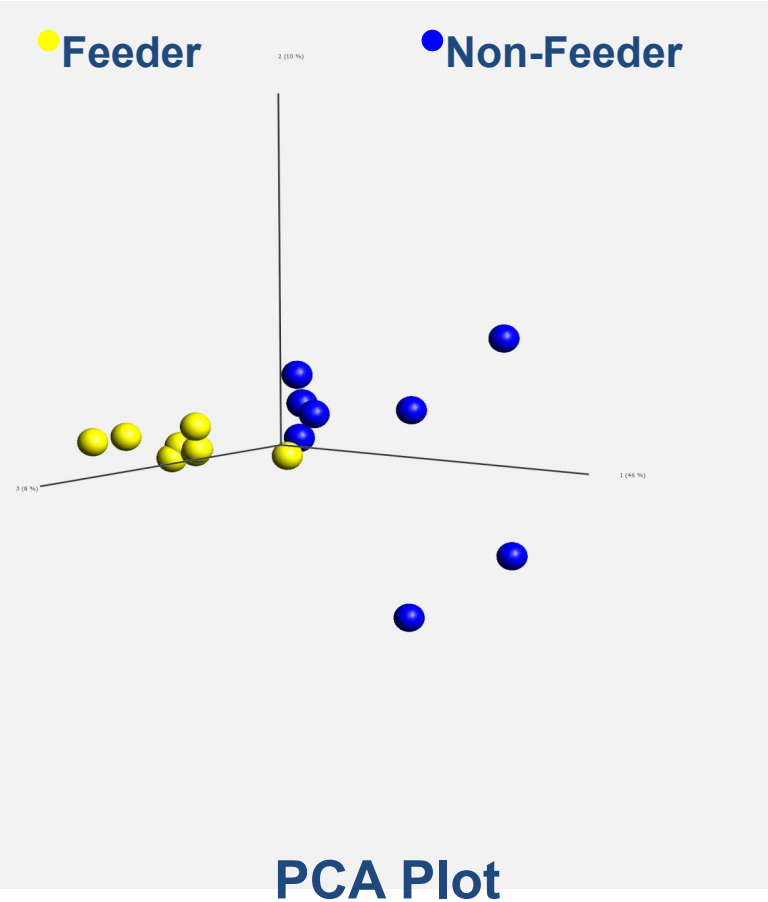
PCA Plot



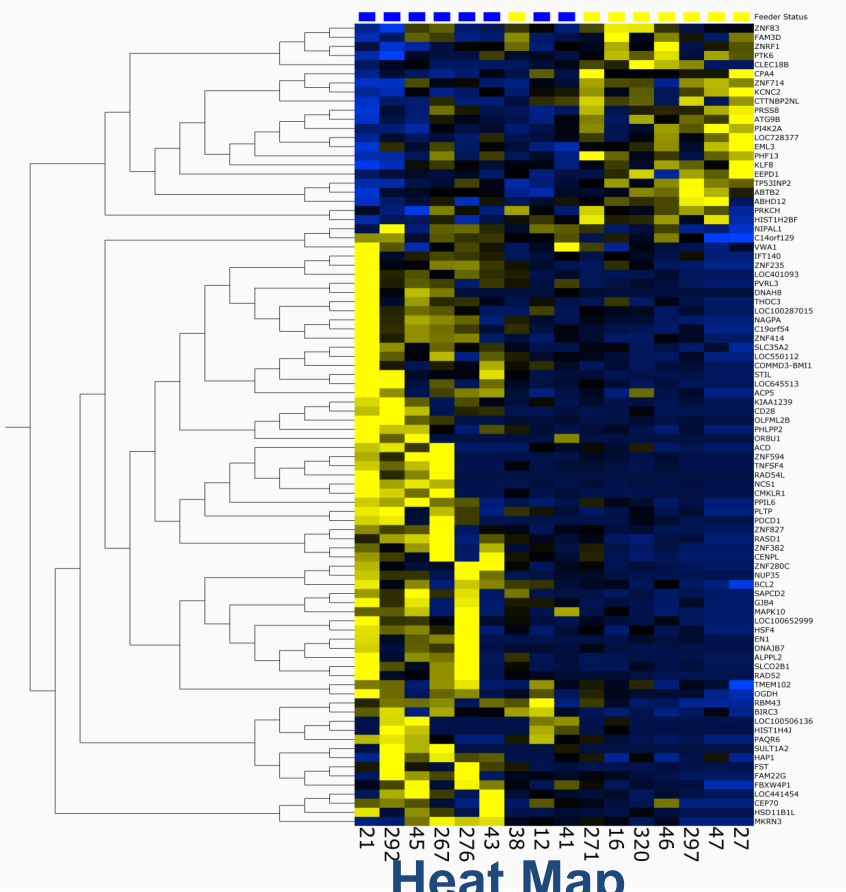
Heat Map

Females

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

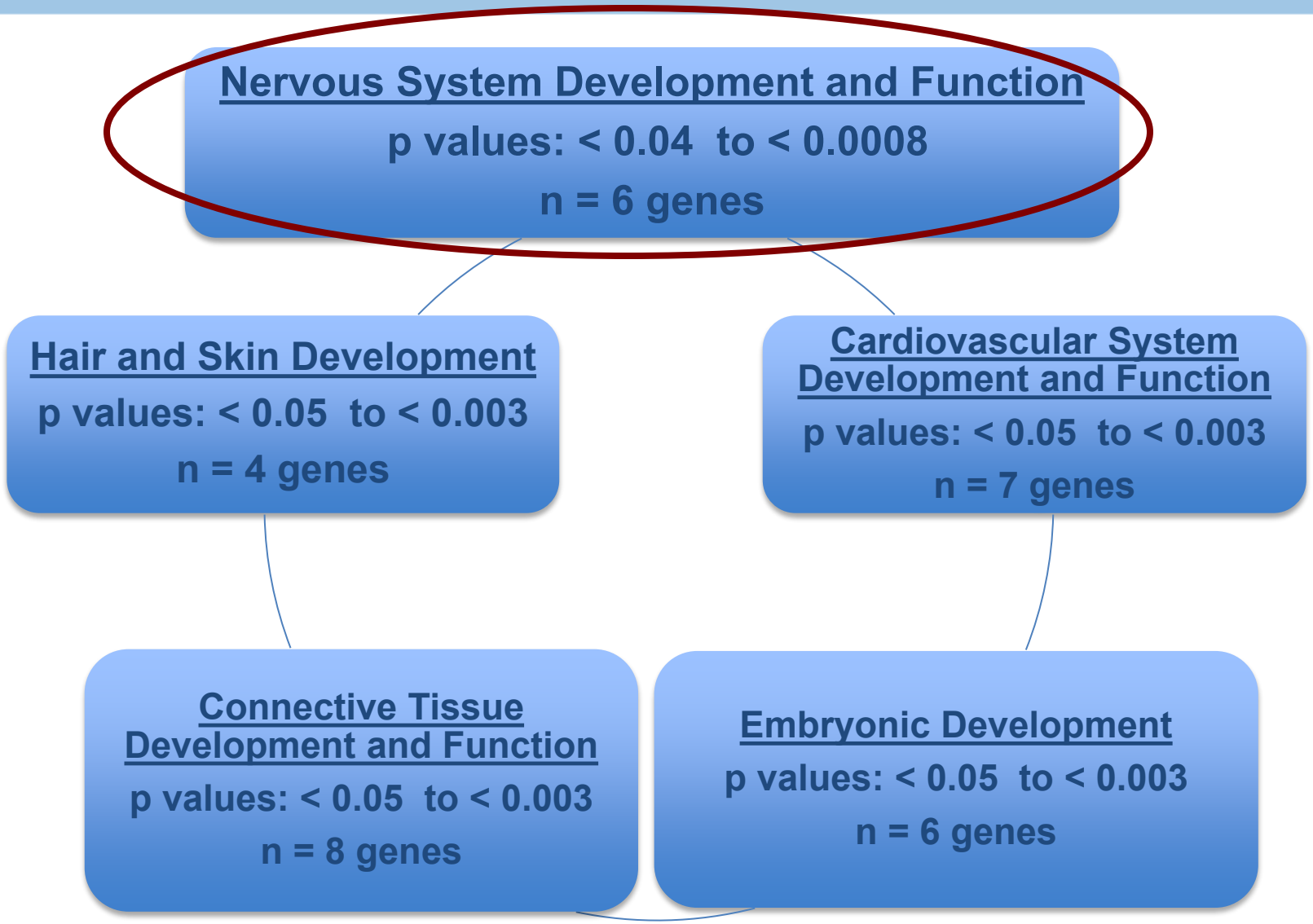


PCA Plot



Heat Map

♂ Disrupted Developmental Pathways



Nervous System Development

- **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**

Ji *et al., Learn Mem* 2008
Bartsch *et al.*, *PNAS 2011*

Expanded NOuRISH Platform

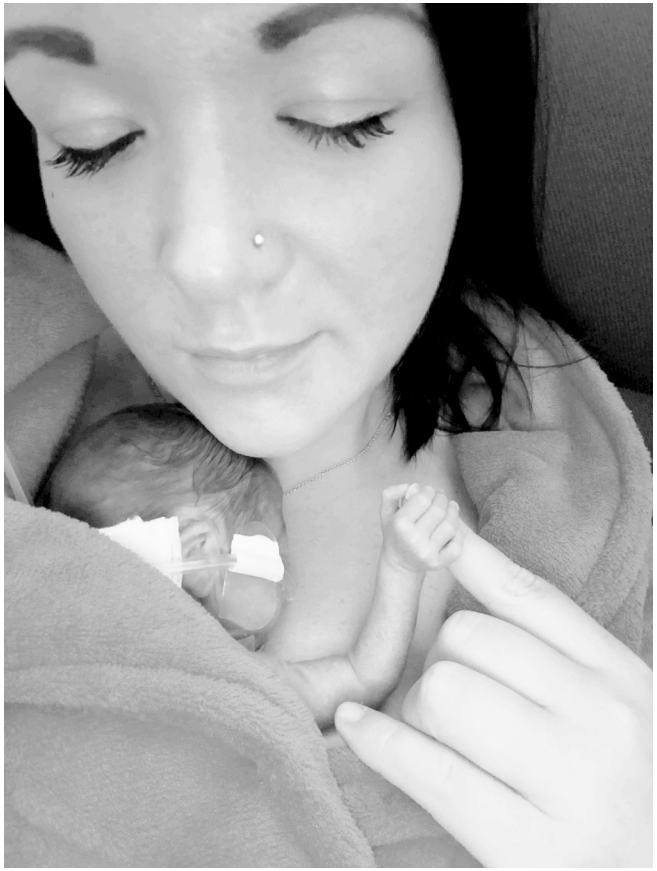
- Incorporating additional genes onto the NOuRISH Platform based on subsequent analysis on the RNA-Seq platform
- Genes related to hippocampal development and memory will now be included
- Hypothesize that expression of these genes may discern between babies that would benefit from the NTrainer and those who require a different therapy

Next Steps

- 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

Olivia

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
- Current and Recent Funding Sources:

NIH NICHD

Charles H. Hood FOUNDATION