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 <u>objective</u> assessment tools for neonatal oral feeding abilities**

Clinical Dilemma: Oral Feeding

- **<u>Majority</u> of premature infants do not have the developmental maturity to successfully and safely feed by mouth**

Photo: Adventure_Photo

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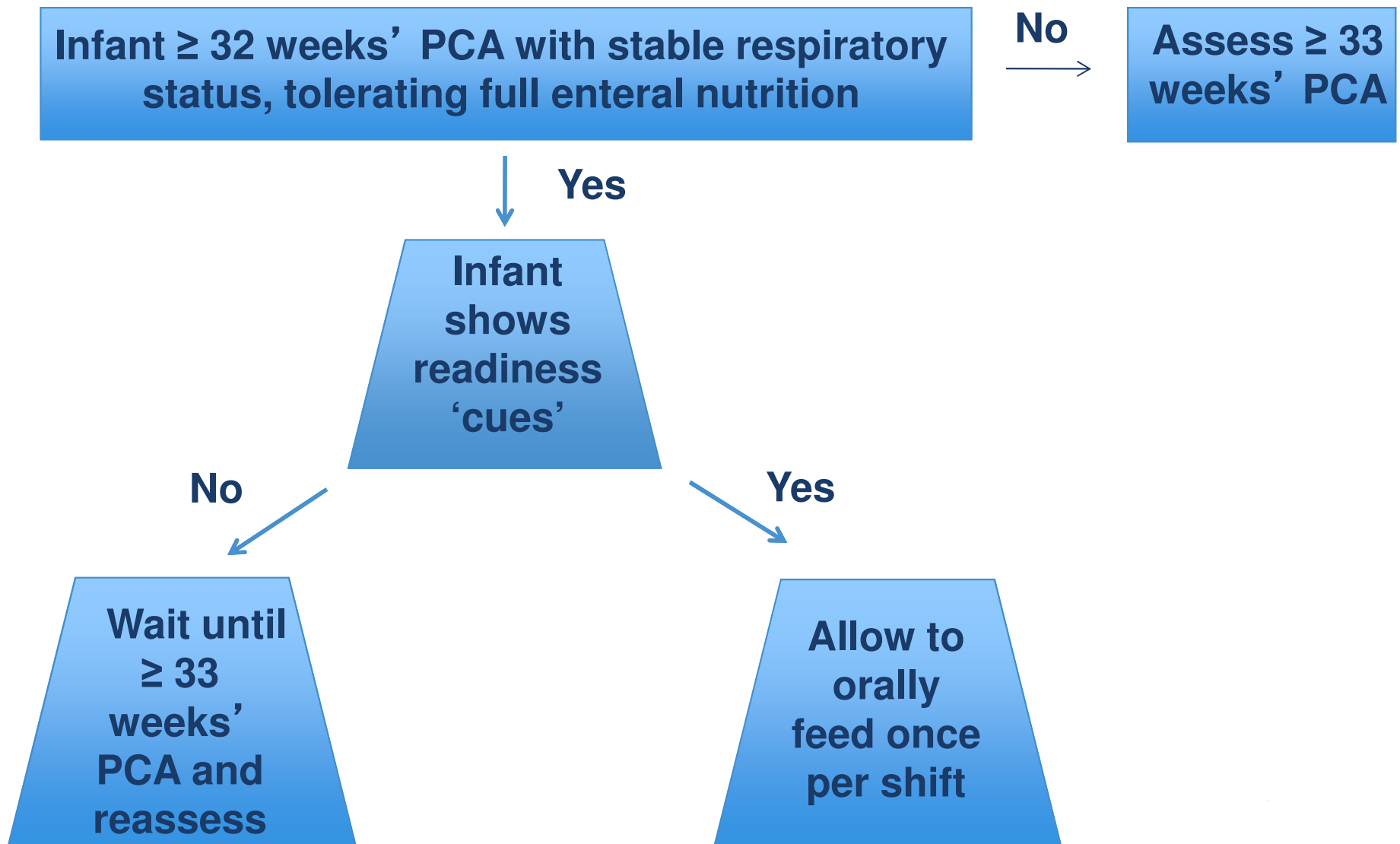

Photo: Christian Wheatley

Oral Feeding

- **The <u>most complex neurological task</u> 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 <u>subjective assessment</u> to determine oral feeding readiness in the newborn:**
  - **Nursing**
  - **Physical, occupational and speech therapists**
  - **Cue-based feeding algorithms**

Current Cue-Based Feeding Tools

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

No → Assess ≥ 33 weeks' PCA

Yes

Infant shows readiness 'cues'

No → Wait until ≥ 33 weeks' PCA and reassess

Yes → Allow to orally feed once per shift

Ludwig SM et al. *Newborn Infant Nurs Rev.* 2007;(7):155–160.

Cochrane Review 2012

- **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**

Crow L et al. *Cochrane Database Syst Rev*. 2012 Apr 18;4:CD005586.

Neonatal Oral Feeding

Failed feeding attempts

- Feeding babies we shouldn't and not feeding babies we could

- Estimated <u>40%</u> 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

McCain GC et al. *J Pediatr.* 2001;139(3):374-379.

Oral Feeding and Outcomes

- <u>Failure</u> 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 <u>first neurological assessment of the newborn</u>

Tsai SW et al. *Pediatr Int.* 2010;52:65-88.
Mizuno K et al. *Dev Med Child Neurol.* 2005;47:299-304.

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 <u>objective</u> oral feeding assessment assay?**

- **<u>Babies do not feed for different reasons</u>**

- **We need to be able to objectively monitor multiple developmental systems <u>simultaneously</u>**
    - **Oral motor control and facial development**
    - **Sensory integration (olfactory, vision, hearing, taste)**
    - **Hunger signaling**
    - **Neurodevelopment**
    - **Gastrointestinal development**

Neonatal Salivary Analysis

My laboratory hypothesized that <u>neonatal salivary gene expression</u> analysis would provide novel, comprehensive and objective evidence about an infant's readiness to orally feed

Photo: Dizzo

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**

Salivary Analysis of the Newborn

- **Analysis of neonatal saliva is not novel**

  – **Proteins — ie, cortisol levels and stress response**

  – **Microorganisms — ie, neonatal salivary CMV studies**

- **It was <u>novel</u> 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**

Neonatal Salivary Transcriptome

First, we had to address technical considerations

1.) <u>Can we do it?</u>

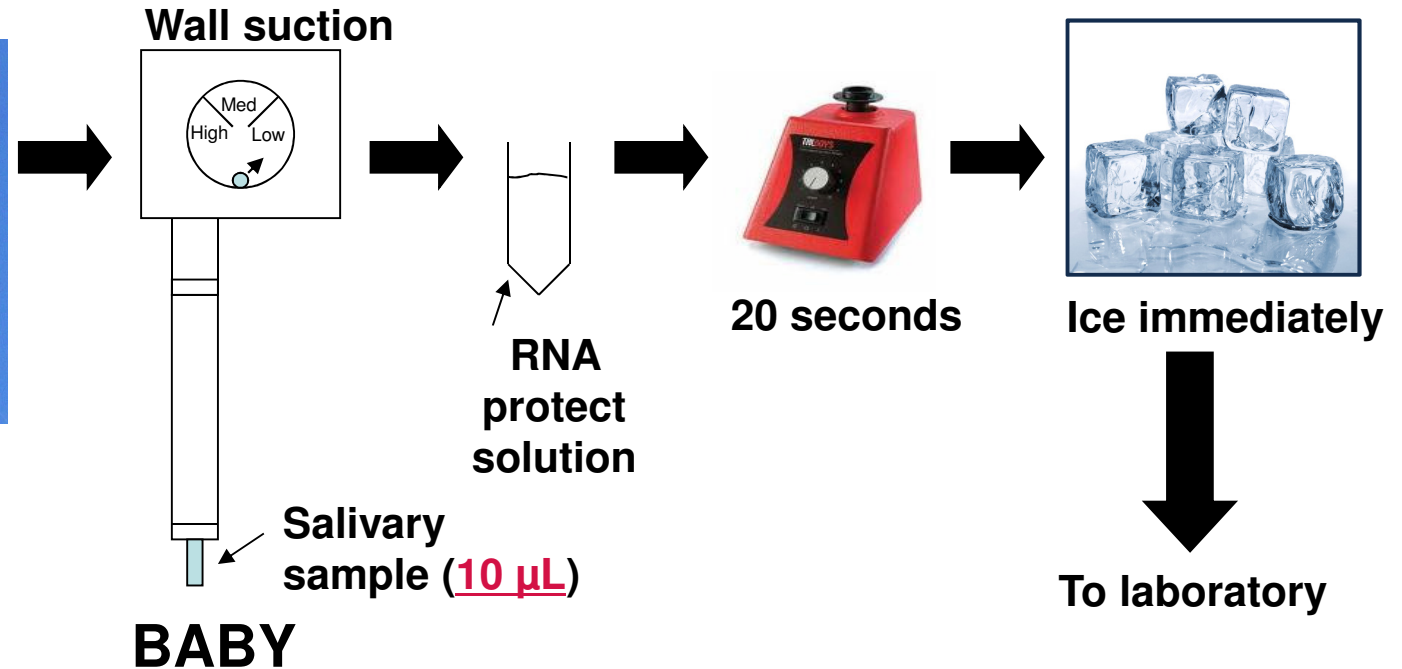   Assays were not developed for neonates

2.) <u>Would it be informative?</u>

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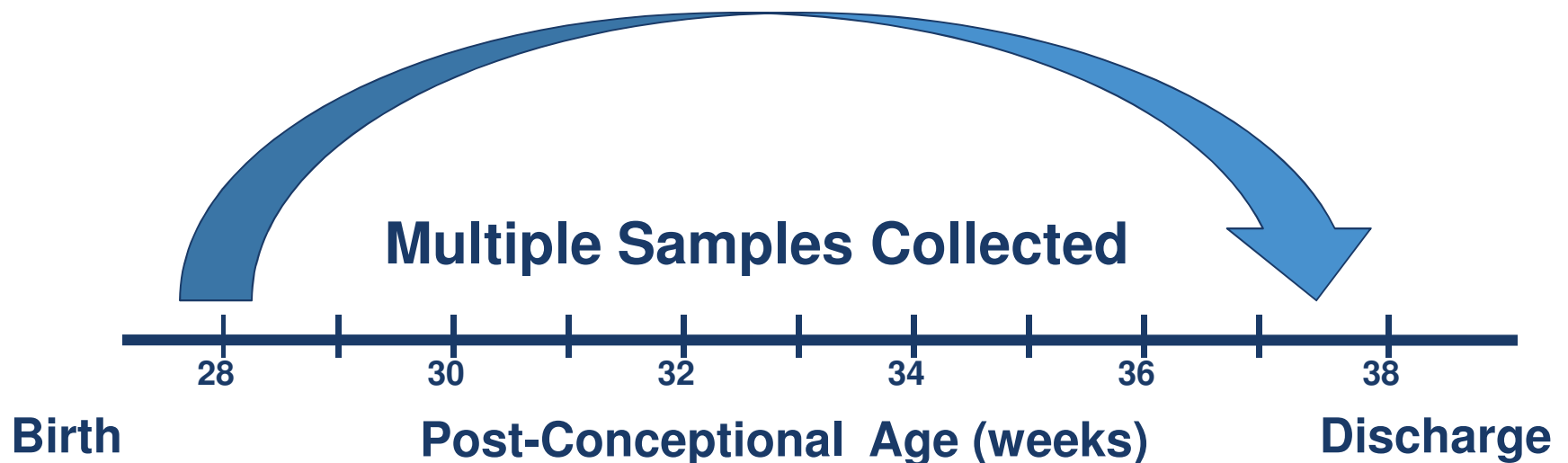
Saliva Collection

1 mL syringe

Wall suction

Med
High   Low

BABY

Salivary sample (10 μL)

RNA protect solution

20 seconds

Ice immediately

To laboratory

Samples are stable at 4ºC for up to 4 weeks before processing

Birth weight: 500 gm to ≥4000 gm

Images courtesy Jill L. Maron, MD, MPH

Initial Salivary Study

- **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

Multiple Samples Collected

28	30	32	34	36	38

Birth   **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)**

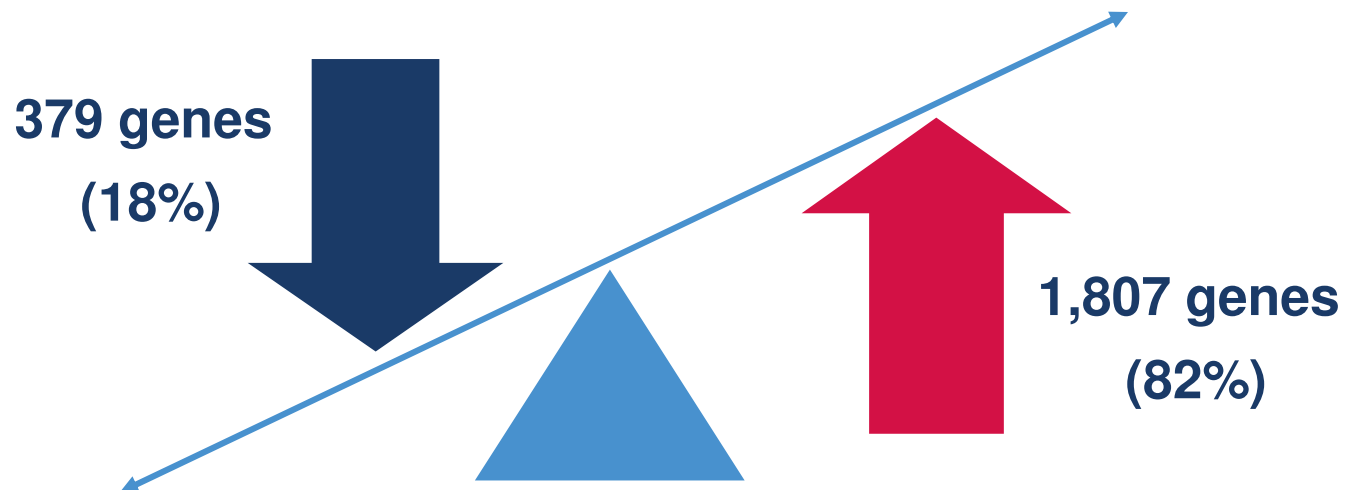- **Each infant served as his/her own control**

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"**
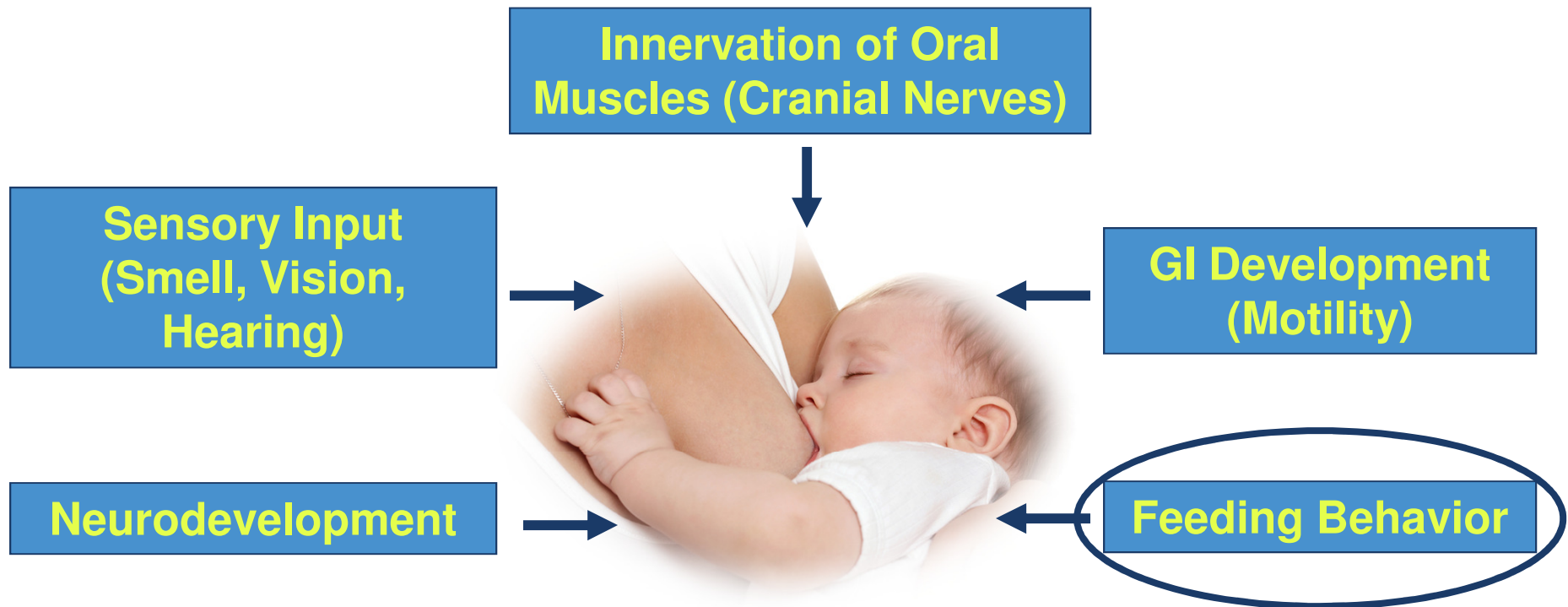
Identifying Oral Feeding Genes

There were 2,186 genes that met criteria and appeared to be related to feeding:

379 genes (18%)

1,807 genes (82%)

Maron JL. *Int J Pediatr*. 2012:195153.

Targeted Analysis

We were able to <u>simultaneously</u> detect genes involved in:

Innervation of Oral Muscles (Cranial Nerves)

Sensory Input (Smell, Vision, Hearing)

GI Development (Motility)

Neurodevelopment

Feeding Behavior

Maron JL. *Int J Pediatr.* 2012:195153.

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**

Neonatal Feeding Behavior

- **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*

Gene of Interest: *NPY2R*

In 1999, Naveilhan and colleagues were the first to generate a knock-out mouse model for *NPY2R*

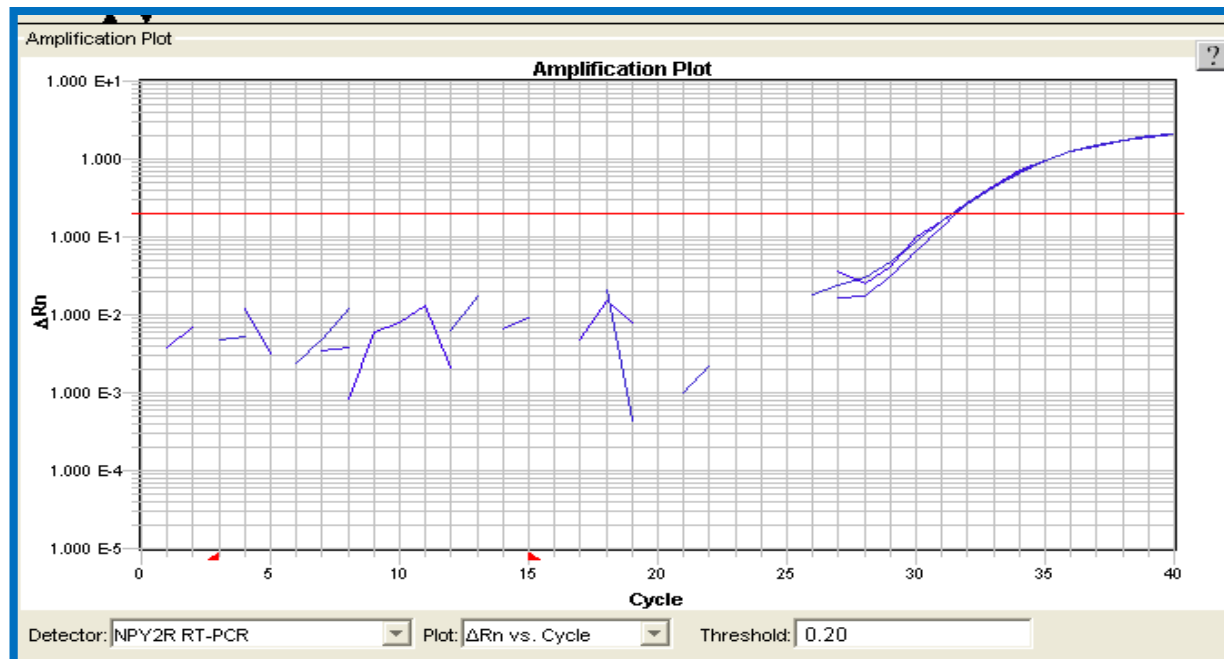**+ *NPY2R* Expression**

ø *NPY2R* Expression

Weighed 180% more than controls

♀ > ♂

Naveilhan P et al. *Nat Med.* 1999;5(10):1188-1193.

Neonatal *NPY2R* Expression

- **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**

Permissions pending

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 <u>95% positive predictive</u> value in determining that an infant cannot sustain full oral feeds**

- **However, the negative predictive value of the assay was only <u>27%</u>**

Maron JL et al. *PLoS One.* **2012;7(5):e37870.**

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

Development of a Diagnostic Assay

- **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**

Systems Biology Approach

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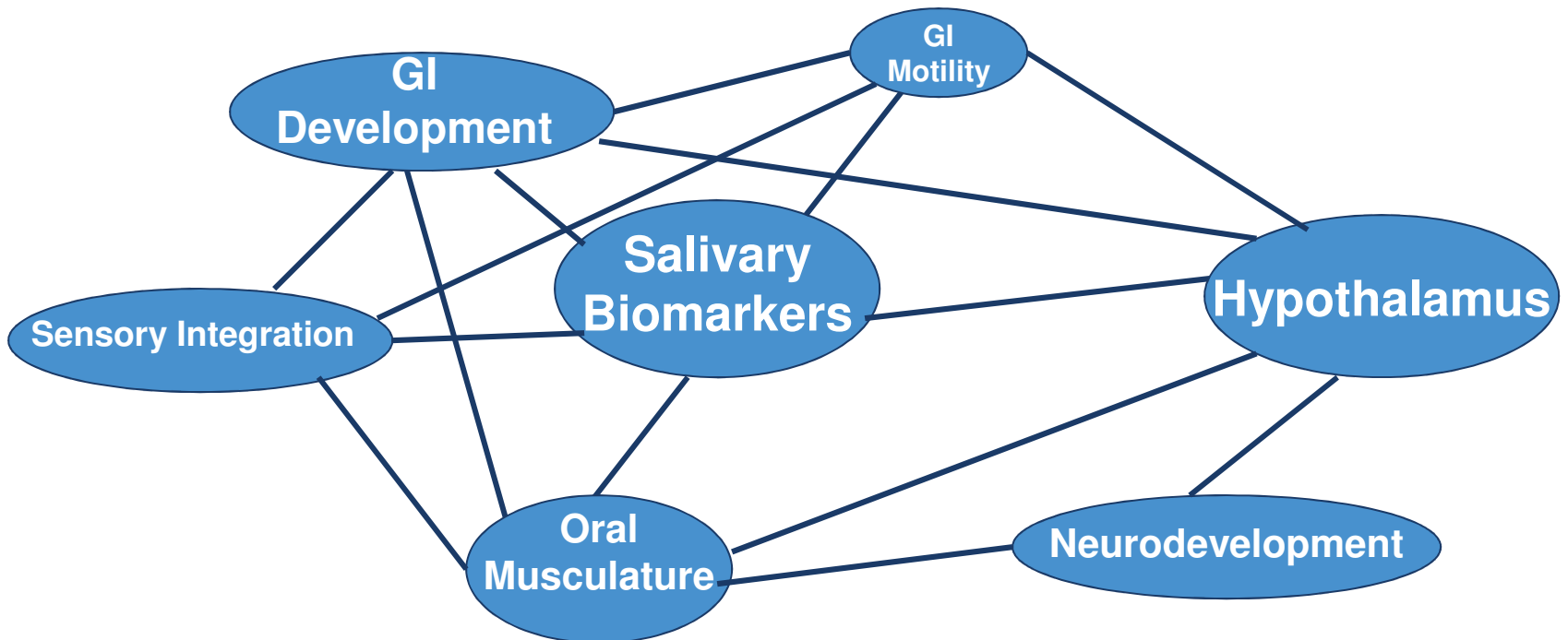
Limitations to Systems Biology

- **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?

Machine Based Learning Algorithm

- **Computational analysis of the microarray data**
- **Identify gene targets in an <u>unbiased fashion</u>**
- **Discover potentially novel genes and gene-gene relationships as they relate to oral feeding in the newborn**
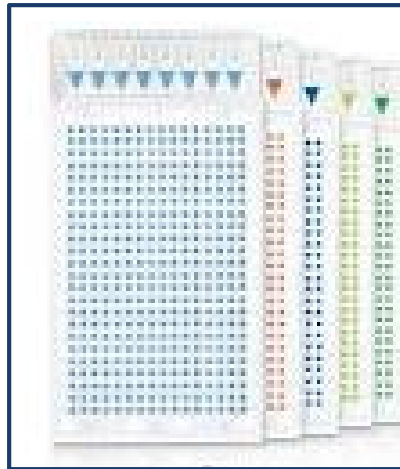
Dr. Gil Alterovitz
Harvard Medical School

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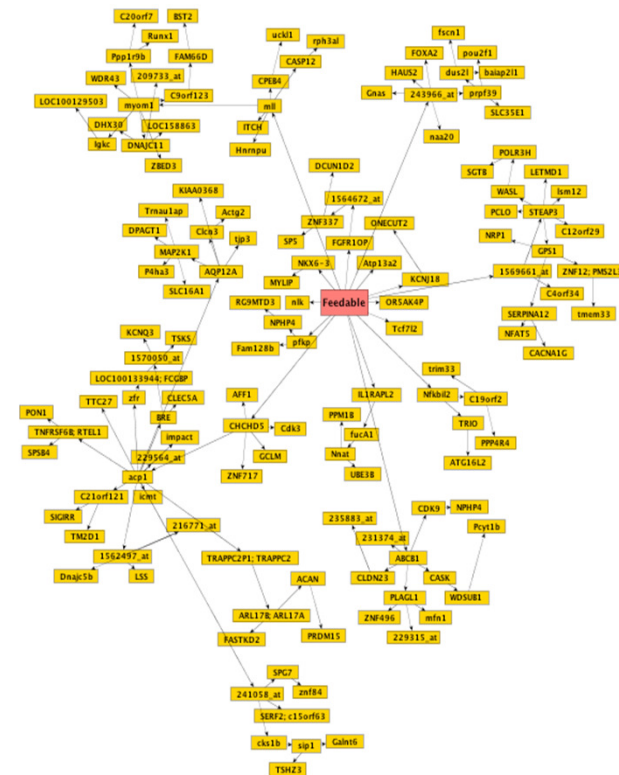
NOuRISH Assay

- **NOuRISH: Neonatal Oral-feeding Readiness in Salivary High-throughput Diagnostics**

- **Custom RT-qPCR assay composed of 24 genes, inclusive of 3 reference genes**

Custom RT-qPCR plates

Images courtesy Jill L. Maron, MD, MPH

NOuRISH Platform

- <u>400</u> 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

Maron JL et al. *J Pediatr*. 2015;166(2):282-8.e5.

NOuRISH Platform-Methods

- **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 <u>succeed</u>

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***

Maron JL et al. *J Pediatr.* 2015;166(2):282-8.e5.

Results

- **After controlling for postconceptional age and sex,
  <u>5</u> 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_

AMPK = Hunger

- **_PLXNA1_:**
  - _Controls axon guidance_
  - _Increased expression in mature compared to developing olfactory sensory neurons_
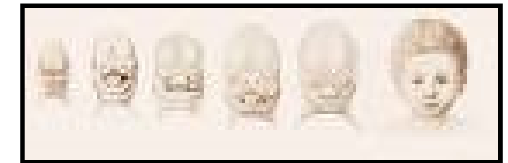
PLXNA1 = Olfactory maturation

McIntyre JC *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

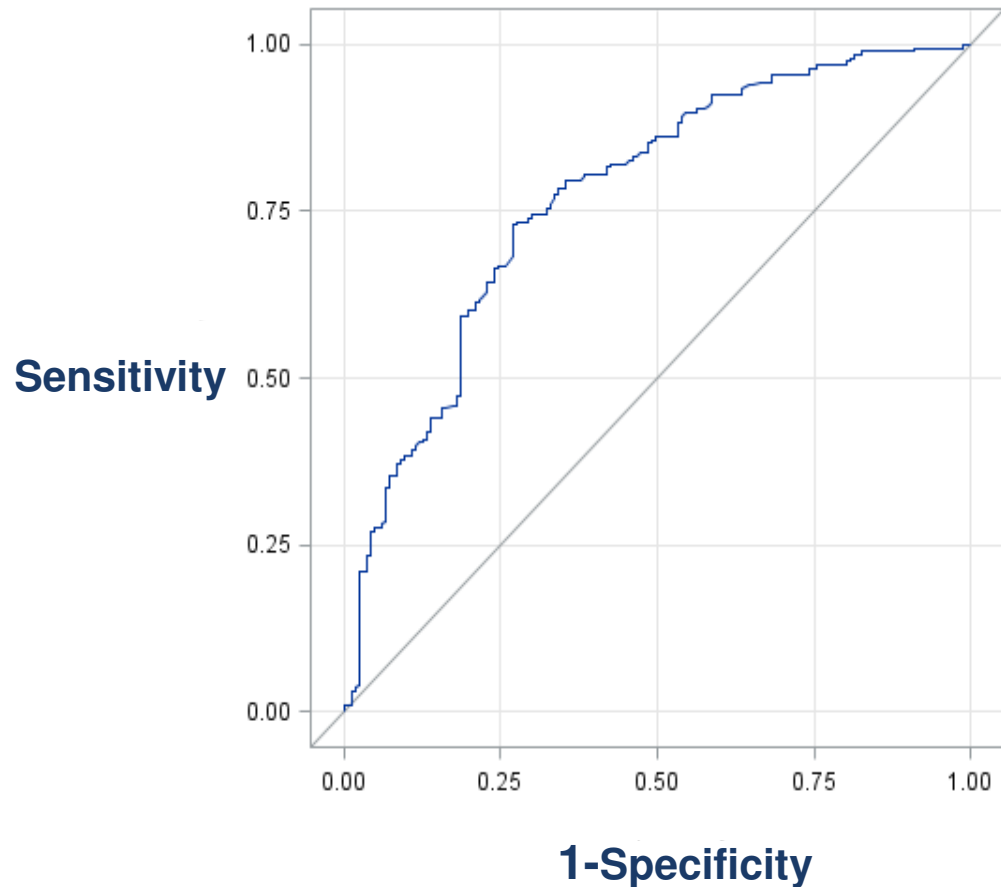| Factors	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Odds Ratio	Odds Ratio 95% CI	P value
PLXNA1	85.05	22.75	56.12	56.72	2.89	(1.47, 5.67)	.002
AMPK	96.36	8.38	55	66.67	3.21	(1.09, 9.48)	.03
WNT3	17.01	72.46	41.77	42.91	0.59	(0.33, 1.07)	.09
NPY2R	39.18	52.69	49.03	42.72	0.71	(0.36, 1.0)	.05
NPHP4	58.25	35.33	51.13	42.14	0.60	(0.34, 1.03)	.06
Age	-	-	-	-	1.43	(1.25, 1.63)	<.001
Sex (Female)	-	-	-	-	1.75	(0.99, 3.06)	.05

Results: NOuRISH Platform

- **Data suggest again that there is no <u>single "magic bullet" biomarker</u> 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**

Maron JL et al. *J Pediatr.* 2015;166(2):282-8.e5.

Results: AUROC

- **In combination, these 5 markers have <u>very good</u> accuracy at predicting feeding success in the premature newborn**

- **This approach is a <u>significant improvement</u> over 'best guess' estimates currently used in clinical practice**

AUROC = 0.78

Sensitivity

1-Specificity

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%

35 week ♀

NPY2R	WNT3	NPHP4	AMPK − PLXNA1 −	AMPK − PLXNA1 +	AMPK + PLXNA1 −	AMPK + PLXNA1 +
−	−	−	15%	45%	48%	73%
		+	15%	33%	35%	61%
	+	−	9%	33%	36%	62%
		+	15%	23%	25%	49%
+	−	−	9%	33%	36%	62%
		+	9%	23%	25%	49%
	+	−	9%	23%	25%	49%
		+	6%	15%	17%	37%

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

Predictive Modeling: Feeding Success

35 week ♂

NPY2R	WNT3	NPHP4	AMPK			
			−		+	
			PLXNA1		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

Predictive Modeling: Feeding Success

35 week ♂

NPY2R	WNT3	NPHP4	AMPK			
			−		+	
			PLXNA1		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

Reprinted from Maron JL et al. Computational gene expression modeling identifies salivary biomarker analysis that predict oral feeding readiness in the newborn. *J Pediatr.* 2015;166(2):282-8.e5. Copyright 2015, with permission from Elsevier.

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 <u>human</u> <u>translational</u> 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**

Feeding and Speech Language Development

- **Collaboration with Dr. Emily Zimmerman, speech pathologist at Northeastern University**

- **Targeting potential biomarkers linking <u>oral feeding maturation</u> with <u>speech language emergence</u>**

- **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**

Fisher SE et al. *Nat Genet.* 1998;18(2):168-170.

FOXP2

- **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

Sex	GA	Birth weight (g)	PCA at salivary collection	PCA at full oral feeds
Male = 13 Female = 7	32.33 (1.04)	1.858 (289)	33.44 (0.78)	35.03 (1.27)

*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.*

Case Report

- "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."

Case Report

- "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**

Acknowledgments

Questions?

Photo: Dizzo

Floating Hospital for Children
at **Tufts** Medical Center

Mother Infant Research Institute