The Adaptability of Milk Molecules: Maternal-Infant Interactions

Culture of Breastfeeding Conference
February, 2016
The question

• Does human milk volume and composition through programmed and patterned neuroendocrine pathways/reflexes and independently of maternal-infant sensorineural and behavioral interactions?
What we know

• Stress can inhibit the oxytocin surge and milk let down can be impaired

• Lactating mothers can produce volumes of milk when they think of their baby, hear their baby cry, etc.
Infants can differentiate milk by smell

- Infants “choose” their own mothers’ milk, turning toward the milk produced by their mother and averting from other mothers’ milk.
In the Wild

• Animal mothers and infants find each other even in the presence of thousands of distracting smells.
So production and volume are affected by sensorineural dyadic patterning.

But what about milk composition? The molecules in milk? Is milk a “designer product”?
Study 1: Postpartum Term Mothers and Infants

Associations between **human milk** SlgA and maternal immune, infectious, endocrine, and stress variables.

**Neuroendocrine and immune relationships in postpartum fatigue.**
*Groër M*, Davis M, Casey K, Short B, Smith K, Groër S.
Stress and Fatigue in the Postpartum

• Stress
  – Roles
  – Physical changes
  – The new baby
  – Relationships

• Fatigue
  – Lack of adequate and continuous sleep
  – Physical exhaustion
  – Daytime sleepiness
  – Metabolic demands
Does this effect milk molecules?
Milk hormones

- **Many hormones** in human milk; function of most is unknown
- Some may be passenger molecules from blood; some may be actively transported.
- Milk Prolactin is associated with immune and neurological development in rodents
- Milk Melatonin in some animal species may play role in regulating infant sleep cycles
Transforming Healthcare Transforming Lives
Postpartum Fatigue

- Is a stressor
- Increases risk for mastitis
- Decreases oxytocin and prolactin
- Associated with postpartum depression
Hormonal Relationships

- Inhibition of dopamine release by melatonin has been demonstrated in specific areas of the mammalian central nervous system (hypothalamus, hippocampus, medulla-pons, and retina).

Diagram:
- Tryptophan
- Melatonin
- Dopamine
- Prolactin and Oxytocin
Melatonin

• Rises in sleep
• Daytime levels higher in people who have had poor sleep
• A potential timekeeper of circadian rhythm in some animals (Asher et al., "Chrono-functional milk": The difference between melatonin concentrations in night-milk versus day-milk under different night illumination conditions. Chronobiol Int. 2015;32(10):1409-16.)
Melatonin

• Few studies of melatonin in human milk
Participants

- 45 exclusively lactating women studied at 4-6 wks postpartum
  - Mean age 28
  - Mean parity 1.8
  - Mean Income $25,000
  - 91% Caucasian
Instruments

- Profile of Mood states (POMS)
- Cohen Perceived Stress Scale
- Epworth Sleepiness Scale
Data Collection

- Hindmilk sample
- Venous blood sample
- Demographics and Instruments

- All collected in the participants’ homes
- $50 honorarium
Prolactin and Melatonin

- Serum and milk prolactin levels were correlated ($r=0.51$, $p=0.01$)
- Lower milk prolactin was associated with higher milk melatonin ($r=-0.56$, $p=0.01$)
- Milk melatonin positively correlated with
  - Epworth Sleepiness scores ($r=0.4$, $p=0.02$) and POMS-fatigue scores ($r=0.44$, $p=0.009$).
Prolactin and Melatonin

• Milk Prolactin inversely correlated with Epworth Sleepiness score \((r=-.49, p=.002)\) and POMS-fatigue scores \((r=-.37, p=.05)\), POMS-confusion \((r=-.51, p=.006)\); Positively correlated with milk sIgA \((r=.41, p=.03)\).
Sleepiness and Milk Melatonin

![Graph showing the relationship between milk melatonin levels and Epworth Sleepiness scale scores. The x-axis represents milk melatonin levels, and the y-axis represents Epworth Sleepiness scale scores. The graph shows a positive correlation, with higher melatonin levels associated with higher sleepiness scores.](image-url)
Conclusions

• The data suggest that milk prolactin and melatonin are associated with stress and fatigue in postpartum breastfeeding mothers

• Is this a dyadic interaction?

• Can babies utilize melatonin in milk?
Study Two
Thibeau, S., D’Apolito, K., Minnick, A., Dietrich, M. Kane, B., Cooley, S., Groer, M.

Relationships of Maternal Stress with Milk Immune Components in African American Mothers of Healthy Term Infants

Methods

• African American mothers of a healthy term infant were given instructions to collect milk (Days 3, 9, and 14) and saliva (Day 9), as well as complete three Perceived Stress Scale questionnaires (Days 3, 9, and 14) and a survey of pregnancy stressors experiences.

• Pearson correlations and linear regressions were performed to assess the relationships of maternal stressors with milk immune components.
Results

• There was at least one statistically significant correlation of a maternal stressor with nine of the 10 milk immune components (effect sizes ranging from $r = 0.22$ to 0.38) on Days 3 and 9. Of all milk immune variables, epidermal growth factor had the most associations with maternal stress indicators. No mediational relationship of cortisol with milk immunity was observed.
For example:

• The environmental stressor of the number of children under mothers’ care was inversely correlated with MCP-1 on Day 3 and IL-6 and TNF-α on Day 9
Study Three

The Impact of Promoter Polymorphisms on Cytokine Concentration in Preterm Breast Milk and Subsequent Infant Outcomes. Journal of Human Lactation (in revision).
Purpose

• to examine relationships between milk Interleukin genotype and levels of Interleukins in milk from mothers of VLBWs (N=63)
Methods

- A study was conducted among mothers (n=63) who delivered very low birthweight infants (n=74, including multiples). Maternal DNA was extracted from breast milk and genotyped using TaqMan.

-
Results

• Multivariate analysis showed trending relationships between maternal IL-6 and IL-10 SNPS and levels in the milk, as well as fecal calprotectin and IVH
Study Four (pending)
Milk has a microbiome

- **microbiome in human milk**, the first food introduced into the gastrointestinal tract, and which may orchestrate, program and time the future development of the communities of microbes living in the child’s gut.

- Signature gut microbiome develops early
Components of the milk microbiome

- $10^3$ to $10^4$ colony forming units in every ml of human milk.
- Most frequently cultured bacteria in human milk are: Staphylococcus, Streptococcus, Lactotoccus, Weissella, Enterococcus, Propionibacterium, Lactobacillus, and Bifidobacterium.
- NGS has identified far more diversity.
Breastmilk

• “lean”-promoting microbiota: Increased Bacterioides, decreased Firmicutes
Influences

• Little known about what influences milk microbiota
  – Specific microbial genera were reported to change over the time of lactation
  – Obese women had less diversity and a different microbiome compared to lean mothers
  – Microbiome changes over time and different dependent upon whether mother is exclusively or partially breastfeeding
Source of Microbes

- Most from maternal GI tract
- One idea is that dendritic cells sample, engulf, and transport bacteria and home to the lactating breast
- Another idea is that bacteria come from infant’s mouth
Milk Biochemistry

- More gut Bifidobacteria (lactobacilli) 60-90% in breastfed; ~50% in Formula fed; FF also have higher Bacteroides, Clostridia, Enterococcus, Staphylococcus
- Rich diversity of HMOs...exceeding other species by up to 100%..promote Bifidobacteria
- BF babies gut microbiota contain bacteria specialized to metabolize HMOs
Study Five
The MOM study
R21 study funded by NIH

• Maureen Groer, Terri Ashmeade, Allyson Duffy, Shannon Morse, Bradley Kane, Shaun Cooley, Ming Ji
Introduction

Human milk is recommended for preterm infants, and is protective against several neonatal illnesses. The immune composition of preterm milk (PTM) differs in several ways from term milk (TM). Little is known about regulation of cytokines, chemokines and growth factors (CCGF) and secretory Immunoglobulin A (sIgA) in PTM. Roles of various CCGFs include infant gut maturation, contribution to microbiome development, infant immune function, and many other undiscovered roles.
Research Questions

• What is the natural course of CCGF and sIgA in human milk across a NICU stay in mother-preterm infant dyads?

• What are relationships between maternal and infant factors and milk CCGF and sIgA?
Sample

- 76 VLBWs, mean weight 1077 Gms, GA 28 weeks; followed for 6 weeks in NICU
- PTM 0.5 ml aliquots collected daily, pooled weekly and assayed for IL-4, IL-6, IL-10, TNF-α, MCP-1, MIP-1α, IP-10, IL-8, EGF by MagPix. Daily volumes of PDM, PTF recorded
- sIgA measured by ELISA (ALPCO)
- Fecal Calprotectin measured weekly in stool (CalPrest)
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Table 2  Infant Characteristics

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<tr>
<th>Infant Characteristics</th>
<th>Values (S.D.)</th>
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<tr>
<td>Infant Birth Weight (Grams) (n=74)</td>
<td>1077.7±219.5</td>
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<td>Apgar at 1 minute (n=74)</td>
<td>6.00±1.92</td>
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<td>Apgar at 5 minutes (n=74)</td>
<td>7.47±1.52</td>
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<td>Gestational Age (n=76)</td>
<td>28.35±2.39</td>
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<td>Score for Neonatal Acute Physiology-Perinatal</td>
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<td>Extension (SNAPPE-Il ) (n=74)</td>
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<td>Weight at 6 weeks of age (Grams) (n=64)</td>
<td>1867.22±317.6</td>
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<td>Weight at Discharge (Grams) (n=70)</td>
<td>2695.77±911.5</td>
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<td>Morbidity</td>
<td>Number of Infants (N=77)</td>
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<td>PFO or PDA</td>
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<td>IUGR</td>
<td>11 (14.5%)</td>
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<td>ROP</td>
<td>14 (18.4%)</td>
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<td>Sepsis</td>
<td>11 (14.5%)</td>
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<td>IVH</td>
<td>9 (11.8%)</td>
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<td>NEC</td>
<td>3 (3.9%)</td>
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<td>Deaths</td>
<td>2 (2.6%)</td>
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PFO=patent foramen ovale  PDA=patent ductus arteriosus  CLD=chronic lung disease  
IUGR=intrauterine growth restriction  ROP=retinopathy of prematurity  IVH=intraventricular hemorrhage  NEC=necrotizing enterocolitis
The first 60 maternal milk samples were labeled and fed to the infant in order. Then infants were fed freshly pumped milk when available, then thawed and prepared frozen MOM. They were further supplemented with varying amounts of HMF, PDM and PTF.
Cytokines
Donor Milk Cytokines

The graph shows the mean cytokine levels in donor milk over different weeks. The cytokines are represented as follows:
- IL-10
- IL-4
- IL-6
- TNF-α

The data is presented for weeks 1 to 6, with each week indicating the number of samples (N). The vertical bars represent the mean cytokine levels in pg/ml, with error bars indicating the variability.
Chemokines and Growth Factors
Donor Milk Chemokines and Growth Factors

![Graph showing log of means (pg/ml) for EGF, IL8, IP10, MCP1, and MIP1 over weeks 1 to 6 and donor milk.](image)
Immune factors

• Secretory Immunoglobulin A is 5 times HIGHER in preterm compared to term milk

• Cytokines, Chemokines and Growth factors have different levels in preterm milk compared to term milk
Variability of CCGF
Trajectory Analysis
Maternal and Infant Factors
Gender
Maternal BMI
Study Five
High PTM cytokines in a mother with liver failure
Conclusions
Maternal and infant factors appear to play some roles in the levels of milk molecules.

- Stress
- Fatigue
- Genetics
- Illness
- Maturational stage
What we don’t know

• How critical are these relationships?
• How can normal breastfeeding be supported to support the most optimal immunity in milk?
• Should milk sharing be done if dyadic influences are critical?
• Should milk pumped in the morning be used in the morning?
Support

- Some of these projects were supported by NIH grants R01NR05000 and R21NR013094.
- We are now following the stol microbiome in VLBW infants at 2 and 4 years of age in R01NR015446 to answer another critical question: Is there an influence of milk volume and immunobiology on the gut microbiome, which is now believed to be a profound influence on health and behavior.
Study Six
The Preterm Infant Microbiome: Biological, Behavioral and Health Outcomes at 2 and 4 years of Age

• R01NR015446
Bacteria are Us

Bacteria have inhabited the earth for at least two and a half billion years. Our evolutionary ancestors arrived in a world dominated by microbes, and, as we evolved, so did they.
• 100 trillion beneficial microorganisms—bacteria, fungi, and viruses—populate the body and are necessary for health
Commensals vs Pathogens

• The beneficial commensals must be recognized and tolerated by the immune system
• The virulent pathogens must be attacked by the immune system
• The immune system is shaped by early life exposures to microbial life
These microbes provide the pioneer culture for the development of the commensal gut microbiome.
Commensals

• Contain polysaccharide-digesting enzymes that are not present in the human genome
• Dietary polysaccharides as degraded in the gut by bacteria
• Commensals inhibit growth and penetration of pathogens
• Make vitamins
• Tolerize the immune system
Other Commensal Functions

- Direct contact of bacterial cells necessary for development, regulation and response of the immune system.
- Bacteria produce key metabolites that cross into bloodstream.
- Bacteria produce key amino acids (e.g., tryptophan) that can affect levels of serotonin and other neurotransmitters.
- Bacteria have different “metabolic rates” so some are more or less efficient in using the food we eat...can result in obesity.
Commensal growth inhibits ability of virulent pathogens to penetrate gut mucus and epithelium
At Birth

- Old idea was that infants were born sterile
  - Now known that meconium has a microbiota, placenta has a microbiota
    - Infant is born with a small maternally originated (largely vaginal) microbiota but within hours is heavily colonized
within hours after birth the infant is heavily colonized
The first exposures

- Maternal vaginal, enteric and skin microbes as the infant is delivered vaginally, and immediately placed on the mother's abdomen and allowed to latch
- And then: Breastmilk!
Influences on Infants’ Gut Microbiota

- Caesarean section vs. vaginal birth
- Food (amount, length of breastfeeding)
- Antibiotics
- Infections
- Prenatal exposures
- Gestational age
- Genetics
- Where born
At Birth

- Vaginal birth is associated with development of a different gut microbiome compared to C-section
  - vaginally delivered infants were found to have similar microbiota to their mother's vaginal microbiota and C-section infants harbored bacterial communities similar to skin surface microbiota
  - Current clinical trial of swabbing vaginal secretions across face of C-section newborn
C-section

• Microbes that first colonize the gut are actually SKIN microbes…and not the mother’s skin
Developmental sequence

• Facultative anaerobes: $10^8$-$10^{10}$/gram feces (Enterobacteriaceae, Enterococci, Streptococci, Staphylococci)

• Within days to week strict anaerobes (Bifidobacteria, Clostridia, Bacteroides)

• By age three signature (adult-type) microbiome
Succession

• Pioneer bacteria compete for substrate and adhesion sites (reduce high redox potential and allow anaerobes)

• Strict anaerobes flourish: Produce metabolites, signal molecules, antimicrobial compounds
Nine Main Phyla in infant gut...~1000 different species
The signature microbiome (up to 3000 species)

- Most term infants achieve this around 2-3 years of age
- It remains one's "signature" for life
- Influenced by food, household, diet, antibiotics and other exposures such as pets
The gut microbiome

• Enormous implications for health…and not just gastrointestinal health!
• One’s signature microbiome can ultimately affect how much a person weighs, how they behave, their development, and health in many different physiological systems
How do we measure microbiome communities in milk or fecal samples?
Next generation sequencing

• Amplification of gene coding for 16SrRNA…allows for identification of thousands of different species based on diversity of this gene.
Illumina MiSeq in our lab
The Preterm Infant

• <1500 Gms
ELBW and VLBW develop dysbiosis

- Inflammation
- Increased breakdown of mucus
- Increased permeability of gut epithelial tight junctions
Factors

• C-section or rapid vaginal delivery
Factors

In utero exposures (meconium microbes reflect intrauterine environment)

- Maternal infections
- Maternal antibiotics and steroids
- Maternal microbiome
Factors

• Preterm Infant Formula
• Donor Milk
Factors

- Immaturity of gut
- Immaturity of immune system
Factors

• Invasive Procedures
Factors

• Lack of maternal contact
Factors

• Universal use of antibiotics in VLBWs
Dysbiosis results
Preterm Infant Gut Microbiome

- Very abnormal: low in anaerobes, sparse, staph, enterococci, enterobacter, yeasts
- Each NICU has its own microbiota that is transferred to the infants and contains antibiotic resistant genes (Brooks et al. Microbiome. 2014 Jan 28;2(1):1.)
- Signature microbiome does not develop at same rate as term infant
Microbiome Influences Phenotype

- Obesity
- Autoimmune disease
- Allergy
- ASD
- Depression
Effects of preterm infant dysbiosis

- Gut inflammation
- Sepsis
- NEC
- Catch-up growth (SGA as well as preterm); obesity and the microbiome (dysbiosis: Firmicutes/Bacteroides)
- Long term effects in VLBW: developmental, GI, autoimmune
Gut microbiome interacts with immune system
Microbiome influences Gut Development

Gene networks differentially expressed in exfoliated epithelial cells from breast- and formula-fed infants. Gene expression was determined in exfoliated intestinal epithelial cells from 3-mo-old breast- and formula-fed infants.
Pathogens and Leaky Gut Theory

• If dysbiosis, inflammation and leaky gut can occur
• Toxins, organism, undigested food, medications, metabolites, can leak out
• Immune response produces potential widespread effects...diabetes, asthma, lupus, multiple sclerosis, depression, anxiety, autism
Leaky Gut
Regulation of the microbiota-brain-gut axis is essential for maintaining homeostasis, including that of the CNS.
The germ free mouse

• A model for what happens when an organism does not have a gut microbiome
Germ free mouse incubators at NIH
Behavior in the germ free mouse

- Increased response to stress
- More daring
- Reduced anxiety
- Reduced non-spatial memory
- Altered monamines
- Lack an ability to recognize other mice with whom they interact
- Altered neurotrophin levels
Microbiota can then be manipulated in germ free mice

- When colonization of the intestines of one strain of germ-free mice with bacteria taken from the intestines of another mouse strain: the recipient animals would take on aspects of the donor's personality. Naturally timid mice would become more exploratory, whereas more daring mice would become apprehensive and shy.
- These tendencies suggested that microbial interactions with the brain could induce anxiety and mood disorders.
The household microbiome (Dr. Jack Gilbert, Argonne National lab)(Science. 2014 Aug 29;345(6200):1048-52)

The Home Microbiome Project followed seven families, which included 18 people, 3 dogs and 1 cat, over the course of 6 weeks. The participants in the study swabbed their hands, feet and noses daily to collect a sample of the microbial populations living in and on them. They also sampled surfaces in the house, including doorknobs, light switches, floors and countertops.
Home is where the microbes are

• They found that people substantially affected the microbial communities in a house—when three of the families moved, it took less than a day for the new house to look just like the old one, microbially speaking.
The “home” of the preterm infant for many weeks
Behavior

• Microbiota NEED us to be social
• Gut-Brain axis involved in behavior
Microbiome and ASD

• women who suffer from a high, prolonged fever during pregnancy are up to seven times more likely to have a child with autism.
• 40 to 90 percent of all children with autism suffer from gastrointestinal symptoms
• Dysbiosis has been noted in gut
• Neuroinflammation
• Study of vancomycin Rx reversing Sx
New meaning to “gut feelings”