Early Life Physiological and Psychosocial Stress Imprints Gut Microbiome in Preterm Infants

To investigate the regulation of early life stress by the brain-gut-microbiota signaling mechanism and explore non-invasive microbial and immune-inflammatory predictors of neurodevelopment.
• The U.S. ranks one of the highest in the world for the number of preterm births.  
< 37 weeks of gestation; 10% in 2014

• In NICU, infants are exposed to numerous early life stressors/pain during critical periods of neurodevelopment.

• 40% NICU survivors have at least 1 neurodevelopmental deficit.

• Yet, mechanisms of early life experiences that alter infants’ health outcomes remain largely unknown.
The Brain-Gut-Microbiota Axis

• Components: Central nervous system (CNS), Hypothalamus-pituitary-adrenal (HPA) axis, Sympathetic-parasympathetic autonomic nervous system, Enteric nervous system, and Intestinal microbiota.

• Bidirectional communication network:
  
  **Top-down:** brain to influence the motor, sensory and secretory modalities of the GI tract

  **Bottom-up:** gut to affect brain function (hypothalamus and amygdala).

Video: https://www.youtube.com/watch?v=5DTrENdWvvM
Brain-Gut-Microbiota Signaling System

<table>
<thead>
<tr>
<th>Infant Characteristics</th>
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<tbody>
<tr>
<td>Gestational age/weight/sex</td>
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<tr>
<td>Mode of delivery</td>
</tr>
<tr>
<td>Feeding type</td>
</tr>
<tr>
<td>Antibiotic/probiotic use</td>
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<tr>
<td>Maternal weight/diet</td>
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<table>
<thead>
<tr>
<th>Brain-Gut-Microbiota Signaling</th>
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<tbody>
<tr>
<td>Neuro-endocrine-immune System</td>
</tr>
<tr>
<td>CNS - central nervous system</td>
</tr>
<tr>
<td>HPA axis - cortisol response</td>
</tr>
<tr>
<td>ANS - autonomic nervous system</td>
</tr>
<tr>
<td>ENS - enteric nervous system</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Early Life Experience</th>
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<tbody>
<tr>
<td>Stressful - negative experiences</td>
</tr>
<tr>
<td>Repeated painful procedures</td>
</tr>
<tr>
<td>NICU environment/stressors</td>
</tr>
<tr>
<td>Infection/inflammation</td>
</tr>
<tr>
<td>Maternal-infant separation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gut Microbiome and Gut Immune/Inflammatory Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbiota abundance/diversity</td>
</tr>
<tr>
<td>Fecal cytokines, calprotectin</td>
</tr>
</tbody>
</table>

Top-Down  Bottom-Up

HRV  ENS

Satiate - positive experiences
- Maternal/paternal proximity i.e., skin-to-skin contact, massage, & other multisensory stimulations
- Pain management

Gut Microbiome Patterns in Infants

• Colonization begins with facultative anaerobic organisms, followed by the development of obligate anaerobes, including *Bifidobacterium*, *Bacteroides*, and *Clostridium*.

• Full-term, breast-fed infant serves as the health standard or the “norm” for newborns.

• **Factors**: delivery mode, feeding, medication use, hospital environment, other early life experiences, and host genetics.
Gut Microbiome Patterns in Infants

• *Dysbiosis* of gut microbiota persists during infancy, especially in preterm infants, and then may reach a stable configuration at age 2 - 3 yrs.

• Preterm infants: demonstrate reduced levels of obligate anaerobes.

• Preterm infants: increased levels of facultative anaerobes, i.e., Enterobacteriaceae and Enterococcaceae

• [https://www.youtube.com/watch?v=Pb272zsixSQ](https://www.youtube.com/watch?v=Pb272zsixSQ)
Study Aims

**Aim 1:** Determine preterm infants’ gut microbiome patterns over first 3-4 weeks

**Aim 2:** The linkage of gut microbiome patterns with early life stress/pain

**Aim 3:** The linkage of gut microbiome with neurodevelopmental outcomes.
Methods

• Design: Prospective longitudinal study.

• Setting: Level IV CCMC NICU at two sites, Hartford and Farmington, CT.

• Subjects: Stable preterm infants (26 – 32 weeks gestation), follow-up for 3-4 weeks.
Methods

• IRB approval and obtain consent from parents.

• Early life stress are measured daily.
  NISS: Neonatal Infant Stressor Scale (Newnham, et. al, 2009)

• Feeding types (Mother’s, Donor’s, Formula)

• Neurodevelopmental outcomes, at 36-38 weeks CA.
  NNNS: NICU Neurobehavioral Scale (Lester, et. al, 2004)
Methods

• Stool samples are collected daily starting 0 – 5 postnatal days for 3-4 weeks.

Culture-independent DNA-based Genomic Technologies:

Gut microbiota community profiles are determined by 16S rRNA sequencing and analysis
Investigating the Infant Microbiome

DNA Isolation from Microbial Community within Fecal Sample

PCR Amplification of the V4 Hypervariable Region of the 16S rRNA Gene

Illumina MiSeq Amplicon Sequencing

Quantitative Insights Into Microbial Ecology

Microbial Composition Analyses
## Results from First Cohort

Clinical characteristics of the initial 29 subjects:

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N (%)</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14 (48%)</td>
<td>Gestational Age (wks)</td>
<td>31.3</td>
</tr>
<tr>
<td>Female</td>
<td>15 (52%)</td>
<td>Birth weight (g)</td>
<td>1460</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td>SNAPEII</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>9 (31%)</td>
<td>8.6</td>
<td>10.5</td>
</tr>
<tr>
<td>Non-Hispanic</td>
<td>20 (69%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delivery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal</td>
<td>12 (45%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-section</td>
<td>16 (55%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>22 (75%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>5 (17%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>1 (3%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Workflow and Quality Control of Stool Samples

- 378 samples from 29 subjects
- Remove 24 samples with low DNA concentration
- 25.6 million high quality and chimera free reads
- Remove 10 samples not making 10,000 cutoff
- 10 meconium yields DNA concentration < 1ng/µg
- 345 samples
**Result 1: Microbiome community composition in preterm infants**

- The most abundant phyla:
  - Proteobacteria (54.3%)
  - Firmicutes (39.2%)
  - Bacteroidetes (3.9%)
  - Actinobacteria (2.4%).

- What contributes to changes in the diversity of the microbiome (Linear mixed-effects models):
  - Time (postnatal days)
  - Gender
  - Feeding type (using mother’s breastmilk or not)
3 Microbial Patterns by Individual Infants

Cong, Xu, Janton, Henderson, Matson, McGrath, Maas, Graf, (2016), PloS One
Result 2: Microbiome Patterns and Gender

Cong, Xu, Janton, Henderson, Matson, McGrath, Maas, Graf, (2016), PloS One

\(\alpha\)-diversity Index:

**Left: males**

\((0.48 \pm 0.26)\)

**Right: females**

\((0.58 \pm 0.22)\)

\(P < 0.05\)
Microbiome Communities and Gender (abundance)

Cong, Xu, Janton, Henderson, Matson, McGrath, Maas, Graf (2016)
Result 3: Gut Microbiome and Feeding Types

Infants Taxonomy

1. MBM n=18
2. Non-MBM n=7
3. Mixed n=4

Infants Taxisomy factor (variable): Other, Actinomycetales, Bifidobacteriales, Bacteroidales, Bacillales, Gemellales, Lactobacillales, Clostridiales, Fusobacteriales, Caulobacteriales, Rhizobiales, Rhodobacteriales, Burkholderiales, Campylobacterales, Aeromonadales, Alteromonadales, Enterobacteriales, Oceanospirillales, Pseudomonadales, Pasteurellales, Pseudomonadales, Xanthomonadales

Kingdom
Phylum
Class
Order
Family
Genus
Species
Results 4: Cumulative Pain/Stressors in the NICU

- Data from initial 50 subjects:
  - **Acute pain/Stressor** daily: 23.4 ± 7.2
    diaper change, heel sticks, arterial blood draw
  - **Chronic procedures** daily 5.1 ± 3.2 hours
    PICC in situ, NG tube in situ, CPAP, systemic infection
Developing a new scale measuring cumulative pain/stressors in the NICU

**Accumulated Pain/Stressor Scale (APSS) in NICUs – based on Neonatal Infant Stressor Scale (NISS, Newnham, et al. (2009) in Australia)**

- Focus group study
- National survey
- 68 procedures
- 9 categories: daily care, feeding, imaging, blood draw, peripheral venous access, central venous access, respiratory care, surgeries and major procedures, and infection

Results 5: Pain/Stressors, Gut Microbiome, and Neurobehavioral Outcomes

Cumulative Pain/Stress and Neurobehavioral Outcome

GLM Regression Analysis: NNNS scale

<table>
<thead>
<tr>
<th></th>
<th>NNNS- Stress/Abstinence (p-value)</th>
<th>NNNS- Habituation (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily acute pain/stressors</td>
<td>0.028</td>
<td>0.016</td>
</tr>
<tr>
<td>Daily chronic pain/stressors</td>
<td>0.051</td>
<td>0.005</td>
</tr>
<tr>
<td>Gender</td>
<td>&gt;0.05</td>
<td>0.022</td>
</tr>
<tr>
<td>Birth GA</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Weight</td>
<td>&gt;0.05</td>
<td>0.004</td>
</tr>
<tr>
<td>SNAPPE-II</td>
<td>&gt;0.05</td>
<td>0.041</td>
</tr>
<tr>
<td>Direct BF</td>
<td>&gt;0.05</td>
<td>0.002</td>
</tr>
<tr>
<td>Skin-to-Skin (KC)</td>
<td>&gt;0.05</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Generalized linear mixed models:

• NISS Acute and chronic pain/stressors: significantly associated with infant neurobehavioral outcomes (NNNS- Stress/Abstinence subscale scores), when controlling for birth GA, birth weight, delivery mode, severity of illness, and direct breastfeeding and kangaroo care contacts, p<0.05.

• NISS scores were negatively correlated to NNNS – Habituation subscale scores, p<0.05-0.01.

• **Indicating that infants who experienced more accumulative pain/stressor had worse stress responses at 36-38 weeks CA.**

• **Indicating infants who experienced less painful/stressful procedures had better habituation and regulation responses.**
Linkages of Pain/Stressors, Gut Microbiome, and Neurobehavioral Outcomes –

### Indicators of Microbiota in Different Levels of Pain/Stressors Experienced in the NICU

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Order / Genus</th>
<th>Indicator Value</th>
<th>Phylum</th>
<th>Order / Genus</th>
<th>Indicator Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infants Experienced Low level of Acute Pain/Stressors</td>
<td>Actinobacteria  Bifidobacteriales/Bifidobacterium</td>
<td>0.65**</td>
<td>Bacteroidetes  Bacteroidales/Bacteroides</td>
<td>0.50*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infants Experienced Low level of Chronic Pain/Stressors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infants Experienced High level of Acute Pain/Stressors</td>
<td>Firmicutes  Lactobacillales/Enterococcus</td>
<td>0.78**</td>
<td>Firmicutes  Lactobacillales/Enterococcus</td>
<td>0.85**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Firmicutes  Lactobacillales/other</td>
<td>0.72**</td>
<td>Firmicutes  Lactobacillales/other</td>
<td>0.80**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Firmicutes  Lactobacillales/Granulicatella</td>
<td>0.69**</td>
<td>Firmicutes  Other/other</td>
<td>0.77**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proteobacteria  Enterobacteriales/Pantoea</td>
<td>0.52**</td>
<td>Firmicutes  Lactobacillales/Granulicatella</td>
<td>0.68**</td>
</tr>
</tbody>
</table>

Note: ** p < 0.01; * p < 0.05

### Indicators of Gut Microbiota with NNNS

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Order / Genus</th>
<th>Indicator Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infants with Less (better) NNNS-stress response</td>
<td>Bacteroidetes  Bacteroidales/Bacteroides</td>
<td>0.55**</td>
</tr>
<tr>
<td></td>
<td>Infants with high (worse) NNNS-stress response</td>
<td>Firmicutes  Lactobacillales/other</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proteobacteria  Enterobacteriales/Pantoea</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Firmicutes  Clostridiales/other</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proteobacteria  Aeromonadales/other</td>
</tr>
</tbody>
</table>

Note: ** p < 0.01
Conclusions

• Over the first 30 days of early life, gut microbiome diversity begins low and increases daily after birth.

• Preterm infants’ gut microbiome community is often dominated by Enterics.

• Preterm infants experience numeric acute and prolonged chronic painful/stressful procedures.

• Time (postnatal days of life), feeding, gender, and pain/stress experience affect the composition of the gut microbiome.

• Gut microbiome indicator species may be omic markers of pain/stressors for infant neurodevelopment.
Multidisciplinary Collaborations

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• UConn Dept. of Molecular & Cell Biology
• Connecticut Children’s Medical Center
• Stevenson Fund Support

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Members and Families