Non-invasive Methods for Assessing Nutritional Regulation of Neonatal Gut Gene Expression and Host-Microbe Interactions

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Presentation Outline

• Introduction
  – Benefits of breastfeeding
  – Factors affecting development of the gut microbiome

• Non-invasive Detection of Intestinal Epithelial Gene Expression
  – Experimental Approach
  – Impact of infant diet on infant gut epithelial gene expression

• Host-Microbe Interactions in the Neonate

• Conclusions
Human Infants are Vulnerable to Nutritional Insults

“Infancy is a uniquely vulnerable period of rapid growth and development and, as such, feeding changes have the potential to impart benefit or harm in the short term, into early childhood, and even later into adulthood”

Pediatric Nutrition

• Proper nutrition is critical for health, growth, and development

• Human milk is the ideal nutrition for infants because it provides all necessary nutrients for normal growth and development and reduces risk of many diseases (American Academy of Pediatrics, 2012)

• Pediatric nutrition is not just about providing nutrients
  – Feeding involves social and tactile interactions
  – Human milk contains bioactive components that serve non-nutritional roles, including stimulating development of the gut microbiota
**Infections**

**Protective Effect of BF:**
- Dosage effect
- Interacts with genetic risk and

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Lower Risk</th>
<th>Breastfeeding</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Otitis media&lt;sup&gt;13&lt;/sup&gt;</td>
<td>23</td>
<td>Any</td>
<td>—</td>
</tr>
<tr>
<td>Otitis media&lt;sup&gt;13&lt;/sup&gt;</td>
<td>50</td>
<td>≥3 or 6 mo</td>
<td>Exclusive BF compared with BF 4 to &lt;6 mo&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Recurrent otitis media&lt;sup&gt;15&lt;/sup&gt;</td>
<td>77</td>
<td>Exclusive BF  &gt;6 mo&lt;sup&gt;d&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Upper respiratory tract infection&lt;sup&gt;17&lt;/sup&gt;</td>
<td>63</td>
<td>&gt;6 mo</td>
<td>Exclusive BF</td>
</tr>
<tr>
<td>Lower respiratory tract infection&lt;sup&gt;13&lt;/sup&gt;</td>
<td>72</td>
<td>≥4 mo</td>
<td>Exclusive BF</td>
</tr>
<tr>
<td>Lower respiratory tract infection&lt;sup&gt;15&lt;/sup&gt;</td>
<td>77</td>
<td>Exclusive BF  &gt;6 mo&lt;sup&gt;d&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>Asthma&lt;sup&gt;13&lt;/sup&gt;</td>
<td>40</td>
<td>≥3 mo</td>
<td>Atopic family history</td>
</tr>
<tr>
<td>Asthma&lt;sup&gt;13&lt;/sup&gt;</td>
<td>26</td>
<td>≥3 mo</td>
<td>No atopic family history</td>
</tr>
<tr>
<td>RSV bronchiolitis&lt;sup&gt;16&lt;/sup&gt;</td>
<td>74</td>
<td>&gt;4 mo</td>
<td>Preterm infants</td>
</tr>
<tr>
<td>NEC&lt;sup&gt;19&lt;/sup&gt;</td>
<td>77</td>
<td>NICU stay</td>
<td>Exclusive HM</td>
</tr>
<tr>
<td>Atopic dermatitis&lt;sup&gt;27&lt;/sup&gt;</td>
<td>27</td>
<td>&gt;3 mo</td>
<td>Exclusive BF negative family history</td>
</tr>
<tr>
<td>Atopic dermatitis&lt;sup&gt;27&lt;/sup&gt;</td>
<td>42</td>
<td>&gt;3 mo</td>
<td>Exclusive BF positive family history</td>
</tr>
<tr>
<td>Gastroenteritis&lt;sup&gt;13-14&lt;/sup&gt;</td>
<td>64</td>
<td>Any</td>
<td>—</td>
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<tr>
<td>Inflammatory bowel disease&lt;sup&gt;32&lt;/sup&gt;</td>
<td>31</td>
<td>Any</td>
<td>—</td>
</tr>
<tr>
<td>Obesity&lt;sup&gt;13&lt;/sup&gt;</td>
<td>24</td>
<td>Any</td>
<td>—</td>
</tr>
<tr>
<td>Celiac disease&lt;sup&gt;51&lt;/sup&gt;</td>
<td>52</td>
<td>&gt;2 mo</td>
<td>Gluten exposure when BF</td>
</tr>
<tr>
<td>Type 1 diabetes&lt;sup&gt;13,42&lt;/sup&gt;</td>
<td>30</td>
<td>&gt;3 mo</td>
<td>Exclusive BF</td>
</tr>
<tr>
<td>Type 2 diabetes&lt;sup&gt;13,43&lt;/sup&gt;</td>
<td>40</td>
<td>Any</td>
<td>—</td>
</tr>
<tr>
<td>Leukemia (ALL)&lt;sup&gt;13-46&lt;/sup&gt;</td>
<td>20</td>
<td>&gt;6 mo</td>
<td>—</td>
</tr>
<tr>
<td>Leukemia (AML)&lt;sup&gt;13-45&lt;/sup&gt;</td>
<td>15</td>
<td>&gt;6 mo</td>
<td>—</td>
</tr>
<tr>
<td>SIDS&lt;sup&gt;13&lt;/sup&gt;</td>
<td>36</td>
<td>Any &gt;1 mo</td>
<td>—</td>
</tr>
</tbody>
</table>

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*Pediatrics* 2012; 121: e827-e41.
Human Milk as a Developmental Modulator

- Growth
- Cognitive Development
- Gut Microbiome & Immune Development
- Metabolic programming
Interaction Between GI, Microbiome and Immune Development

Early Nutrition
(Breast vs Formula feeding)

- Establishment of the Microbiome
- Intestinal Development
- Immune Development
Factors Impacting Establishment of the Intestinal Microbiota

- Milk oligosaccharides (HMO)
- Bacteria in milk
- Bacteria on maternal skin

**Term vs. Preterm Delivery**
- **Preterm**: Slower colonization and less diversity

**Route of Delivery**
- **C-section**: less *Bifido* and *Bacteroides*; more *E. coli* & *C. difficile*

**Perinatal Antibiotics**
- Reduced overall diversity and numbers

**Type of Nutrition**
- Milk oligosaccharides (HMO)
- Bacteria in milk
- Bacteria on maternal skin

**Other**
- Siblings, pets in the home, smoking, daycare, etc

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  – Impact of infant diet on infant gut epithelial gene expression

• Host-Microbe Interactions in the Neonate

• Conclusions
Looking into the “Black Box”: Host-Microbe Interactions in the Neonate

What components in the diet affect the intestinal microbiota?

What bacteria and their genes are involved in the interaction?

Which human genes are involved in the interaction and respond to bacterial signals?

Defining how early nutrition regulates gut development in human infants has been limited by the lack of non-invasive approaches suitable for use in healthy human infants.

- Exfoliated intestinal cells may provide a means to investigate the impact of nutrition on intestinal development and function (Davidson et al., 1995)
- Approximately 1/6 to 1/3 of epithelial cells are shed daily (>10^{10} cells/day) (Potten et al., 1979)
Overall Experimental Approach


Host-Microbe Interactions in the Neonate

What components in the diet affect the intestinal microbiota?

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Experimental Subjects

- Vaginally-delivered, term infants of second parity mothers that were medically certified as healthy
- Exclusively breast-fed or fed Enfamil Lipil formula (Mead Johnson, Evansville, IN) until 3 months of age
- Exclusion criteria: formula intolerance, combined breast milk/formula, non-study formula, juice or solid foods

<table>
<thead>
<tr>
<th>Breastfed (BF)</th>
<th>Formula-fed (FF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 16</td>
<td>N = 10</td>
</tr>
<tr>
<td>Maternal Age (years)</td>
<td>29.5 ± 4.2</td>
</tr>
<tr>
<td>Infant Birth Weight (kg)</td>
<td>3.78 ± 0.56</td>
</tr>
<tr>
<td>Infant Birth Length (cm)</td>
<td>52.5 ± 5.5</td>
</tr>
</tbody>
</table>

Milk Intake & Infant Growth

- No significant difference in intake or weight gain
Stool Sample Processing

- Sample was collected at 3 months postnatal age by the parent.
- Freshly voided stool (~10 g) was placed into a sterile tube containing Trizol reagent (Ambion, Austin, TX).
- Poly A+ RNA was isolated to from sloughed epithelial cells to enrich mammalian RNA using established methods (U.S. Patent 6258541).

57,000 genes on array → 4,250 genes showed signal on all arrays → Prior Knowledge 529 genes → 1,214 genes had p-value <0.05 → 146 genes

- These 146 genes were subjected to further analyses:
  - Linear Discriminant Analysis (LDA) – Best Classifiers of BF vs FF
  - Gene Networks (Metacore™, GeneGo, St. Joseph, MI) - Networks
BF vs FF Infants (Gene Classifications)  
“Linear Discriminant Analysis”

2-Gene Combination

Uncoupling Protein 2 vs Endothelial PAS Domain Protein 1

- Formula -Fed
- Breast-fed

3-Gene Combination

Uncoupling Protein 2 vs Synaptophysin vs Forkhead box protein E3

- Formula -Fed
- Breast-fed
## LDA - Best Genes For Classifying BF vs FF

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Function</th>
<th>Fold Change (BF/FF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPAS1</td>
<td>Transcription Factor (TF); cellular response to hypoxia</td>
<td>3.3</td>
</tr>
<tr>
<td>NR5A2</td>
<td>TF, encodes liver receptor homolog-1 (LRH-1); development</td>
<td>2.8</td>
</tr>
<tr>
<td>NR3C1</td>
<td>Encodes glucocorticoid receptor</td>
<td>5.5</td>
</tr>
<tr>
<td>PCDH7</td>
<td>Encodes protocadherin-7; membrane protein</td>
<td>3.9</td>
</tr>
<tr>
<td>ITGB2</td>
<td>Encodes integrin beta-2 (CD18); ICAM-1 receptor</td>
<td>2.5</td>
</tr>
<tr>
<td>FGF5</td>
<td>Encodes fibroblast growth factor 5; mitogenesis &amp; cell survival</td>
<td>2.0</td>
</tr>
<tr>
<td>TJP1</td>
<td>Encodes ZO-1; intercellular tight junctions</td>
<td>2.2</td>
</tr>
<tr>
<td>MYB</td>
<td>TF, transcriptional transactivation; proto-ongene</td>
<td>2.8</td>
</tr>
<tr>
<td>EPIM</td>
<td>Syntaxin 2/Epimorphin; epithelial cell morphogenesis</td>
<td>2.5</td>
</tr>
<tr>
<td>BAD</td>
<td>BCL2-associated agonist of apoptosis</td>
<td>4.0</td>
</tr>
</tbody>
</table>
Metacore™ Gene Networks – BF vs FF Infants

- Signal transduction
  - WNT
  - NOTCH
  - TGF-β

- Cytoskeleton remodeling
  - Cell migration

- Cell adhesion
  - Barrier function

- Immune response
  - Inflammation
  - Histamine

From: Metacore™, GeneGo, St. Joseph, MI
Summary of Intestinal Gene Expression

• The relationships between diet and host gene expression can be assessed non-invasively in the human infant
  • 2- and 3-gene combinations were shown to distinguish BF from FF infants

• Provides insight into potential mechanisms whereby human milk regulates intestinal development and represent potential targets for manipulation of infant formula composition

• In preterm infants, this approach has shown developmental differences in gene expression compared to term infants (Knight et al. 2014)
  - Lower expression of genes in LCPUFA synthesis
  - Lower proliferation/cell cycle gene expression
  - Greater inflammatory gene expression

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Disease

Health

Pyrosequencing of V1-V3 region of 16s rRNA gene amplicons
• 321,822 sequences (10,743 per sample)
• Distance based redundancy analysis (dbRDA) showed that the overall structure of the microbiome differed between BF and FF infants.

Fecal Microbiota of BF and FF Infants

- Sequences classified using Ribosomal Database Project Classifier
  - 7 phyla and 62 genera were identified
- Actinobacteria was the most abundant, but not different in BF and FF
- BF has lower Firmicutes and higher Bacteroidetes than FF

• 5 distinct signatures: FF, BF (3 infants), BF1, BF2, BF3
• Can we use differences in microbiota of BF and FF infants to predict differences in host gene expression?
- SEED level 1 functional categorization via MG-RAST revealed
  - A larger proportion of genes involved in CHO metabolism in FF
  - Lower proportion of genes involved in AA and protein metabolism in FF
  - That virulence characteristics differed between FF and BF babies
Multivariate Analysis of Host Transcriptome and Functionally-Profiled Microbiome Data

A

Stool Sample

Exfoliated host epithelial cells

Poly-A selected host mRNA

Microarray analysis

Host gut transcriptome gene expression data: immunological, intestinal, and other known genes

Infant gut bacteria

Bacterial shotgun DNA

454 sequencing

Metabolic profile (e.g., virulence) and phylogenetic distributions based on sequence data proportions

Repeated Canonical Correlation Analysis (CCA) via gene subsets

Multivariate correlation measures between gene expression and microbiome metabolic characteristics

B

C

D

660 Random genes

459 Intestinal Biology genes

660 Immunity and Defense genes

From: Chapkin et al., 2010

660 Immunity and Defense genes

Virulence Genes

# 11 Baby Immunity & Defense Genes Most Related to Microbial Virulence Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TACR1</td>
<td>Neurokinin (NK) 1 receptor; member of the tachykinin family of G-protein-coupled receptors</td>
</tr>
<tr>
<td>VAV2</td>
<td>Guanine-nucleotide exchange factor</td>
</tr>
<tr>
<td>ALOX5</td>
<td>Lipoxygenase gene; synthesis of leukotrienes from arachidonic acid</td>
</tr>
<tr>
<td>NDST</td>
<td>GlcNAc N-deacetylase/N-sulfotransferase-1; heparin sulfate synthesis</td>
</tr>
<tr>
<td>REL</td>
<td>Member of Rel/NFKB family</td>
</tr>
<tr>
<td>BPILI</td>
<td>Bactericidal/permeability-increasing protein-like 1; LPS binding protein</td>
</tr>
<tr>
<td>AOC3</td>
<td>Mediates the binding of lymphocytes to vascular endothelial cells in an L-selectin-independent fashion</td>
</tr>
<tr>
<td>KLRF1</td>
<td>NK Cell Receptor; stimulates natural kill cell cytotoxicity</td>
</tr>
<tr>
<td>DUOX2</td>
<td>NADPH oxidase; lactoperoxidase-mediated antimicrobial defense</td>
</tr>
<tr>
<td>IL1A</td>
<td>Cytokine secreted by activated macrophages, IL-1 stimulates thymocyte proliferation</td>
</tr>
<tr>
<td>SP2</td>
<td>Transcription factor required for expression of cell cycle- and developmentally-regulated genes</td>
</tr>
</tbody>
</table>

**Up-regulated in BF**

**Down-regulated in BF**
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Summary of Host-Microbe Gene Expression

- We found evidence of multivariate structure relating the host immune system and microbiome virulence characteristics.

- The virulence properties of the microbiota were the most responsive characteristics with respect to BF versus FF, but probably do not reflect an infection.
  - BF babies had a larger complement of gram-negative bacteria than FF.
  - Gram-negative bacteria have genes that, although classified as 'virulent,' can activate the immune system but not cause an infection in the process.

- The relative abundance of CHO and protein metabolizing genes differed in the microbiota of FF and BF infants.

- These data suggest linkages between early nutrition and the functional characteristics of the neonatal microbiota.
Acknowledgments

• Robert Chapkin PhD, Texas A&M University

• Term Infant Study:
  • Rose Ann Mathai, MS, RD
  • Marcia Monaco PhD

• Metagenomics and Bioinformatics:
  • Mei Wang PhD and Min Li PhD
  • Scott Schwartz PhD, Ivan Ivanov PhD and Iddo Friedberg PhD

• HMO Analyses:
  • Shuai Wu and Carlito Lebrilla, PhD

• NIH CA59034, NIH CA129444, NIH DK71707, NIH P30ES09106
• DNS Vision 20/20
• Mead Johnson Nutrition
Breastfeeding: A Balance of Art and Science
Stool Sample Processing

• Sample was collected at 3 months postnatal age by the parent

• Freshly voided stool (~10 g) was placed into a sterile tube containing Trizol reagent (Ambion, Austin, TX)

• Samples were mixed by hand to create a homogenous sample and were immediately frozen at -20 °C

• Samples were held at -80 °C until shipped on dry ice to Texas A&M University

• An additional aliquot was immediately frozen for microbial and SCFA analyses