The Growth of Children and Epigenetic Marks Associated with a Longer Duration of Breastfeeding

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Speaker Introduction

- Heide Temples teaches pediatrics to the graduate students in the Family Nurse Practitioner Program at Clemson University. She completed her PhD in Healthcare Genetics and was a fellow in the Summer Genetics Institute (SGI) at the National Institutes of Health (NIH) in Bethesda, Maryland. She has been an actively practicing pediatric nurse practitioner with over 20 years of clinical experience. She has researched adolescent obesity with the University of South Carolina and was a research nurse at Baylor University Medical Center in Dallas, Texas. Dr. Temples area of expertise is childhood obesity and she is researching the epigenetic influence of breastfeeding on the growth and development of children with the Murdoch Childrens Research Institute in Melbourne, Australia.

Disclosures

- I have no conflicts of interest to disclose.

Learning Objectives

- List the critical time periods of greatest epigenetic change that can influence permanent changes in metabolism modulating childhood obesity.
- Discuss how healthcare providers can tailor education and interventions focused on early life nutritional habits to help prevent childhood obesity.
- Describe the changes in the BMI, arm circumference and abdominal circumference with longer duration of breastfeeding.

Childhood obesity

- This research examines the use of a pluralistic model of heredity which has both genetic and environmental, non-genetic (epigenetic) changes that lead to disease to further identify some of the “missing heritability” in childhood obesity.

Missing heritability

- Genetic component contributes 40-70% to the variation of body mass index (BMI) and obesity.
- Shortcomings in identifying the genes contributing to obesity are referred as the “missing heritability” (Manco, 2013).
- The GWAS studies explain less than 20% of the phenotypic variance in obesity (Wallace, 2010).
Current research

- Current genetic research to identify the “missing heritability” in obesity involves investigating:
  - gene-to-gene interactions
  - rare variants
  - epigenetic factors to fill the gap (Manco, 2013).

Epigenetics?

- The term ‘epigenetics’ literally means ‘above DNA’ and refers to the study of molecular modifications that influence gene activity and chromosome structure (Saffery, 2012).
- The action of environmental compounds inducing epigenetic patterns during key developmental periods influences phenotypic variation, and in some cases leads to diseases, such as obesity (Skinner, 2010).

Epigenetics

- Period of greatest subsequent epigenetic flux is during the critical prenatal and postnatal periods of mammalian development that influence the developmental pathways, inducing permanent changes in metabolism and modulating chronic disease susceptibility (Saffery, 2011, pg 55).

Epigenetic Video

- Professor Vivette Glover, Imperial College, London

Epigenetics

- Epigenetics regulates genome activity and provides a mechanism allowing environmental factors to regulate gene expression and phenotype formation (Skinner, 2010; Guerrero-Bosagna, 2008; Waterland, 2008).
Breastfeeding-Epigenetic Literature Review

- "Molecular biology studies have shown that nutrients, either directly or by hormonal activity, are able to significantly influence the expression of genes" (Verduci, 2014, pg 1712)
- Nutritional epigenetics occurs when the diet changes the gene expression and phenotype formation.
- "Breastfeeding, has been associated with lower risk of being obese and developing type 2 diabetes" (Verduci, 2014, pg 1717)

Breastfeeding-Epigenetic Literature Review

- Plausible mechanisms by which breast-feeding may show a protective role:
  - Nutrients composition of breast milk
  - Feeding behavior associated with breastfeeding
  - Lower protein and energy content in breast milk than formula
  - Bifidobacterium and Lactobacillus spp., growing in gut microbiota
  - Self-regulate of breast-fed infants
  - Hormone-like compound, e.g., ghrelin and leptin
  - Nutritional epigenetics (Verduci, 2014)

Nutritional-Epigenetics Literature Review

- The risk of developing obesity depends on the interaction between genotype and individual lifestyles,
- Epigenetic regulation of specific genes may also become crucial in determining the individual risk for obesity (Verduci, 2014)
- The specific genes examined in this literature review are the PPARγ2 gene, LEP gene and the IGF2/H19 gene.

Breastfeeding-Epigenetic Literature Review

- The peroxisome proliferator-activated receptor-γ (PPARγ2) transcription factor is primarily expressed in adipocytes
- Nuclear hormone receptor family influencing whole body energy homeostasis
- Pro12Ala substitution at codon 12 polymorphism has been shown to be associated with higher BMI, waist circumference, and obesity risk (Verduci, 2014, pg 1717).

Breastfeeding Epigenetic Literature Review

- However, this association was not seen in Ala 12 carriers who had been breast-fed (even for a short period).
- Breastfeeding may have beneficial effect on the obesity risk later in life in genetically predisposed groups
- Breast milk may have an epigenetic effect associated to the adiposity and development of related disorders (Verduci, 2014)
Breastfeeding-Epigenetic Literature Review
- Methylation of the LEP gene in children at 17 months of age in association with early life nutritional factors
- The LEP gene is highly associated with obesity and insulin resistance
- Ninety-nine mother and child pairs were included in their breastfeeding study
- 75 were breastfed
  - 14 were breastfed < 1 month
  - 22 were breastfed for 1-3 months
  - 21 were breastfed for 3-6 months
  - 18 were breastfed for more than 6 months (Obermann-Borst, 2013).

Breastfeeding-Epigenetic Literature Review
- Methylation of LEP and the duration of breastfeeding, birth weight, BMI, gender, leptin concentration in the child and maternal education
- Duration of breastfeeding was negatively association with LEP methylation
- Lower methylation of LEP leads to increased gene expression and higher concentrations of serum leptin
- Leptin is a neuroendocrine appetite regulator and is present in breast milk but not in formula (Obermann-Borst, 2013, pg 3).

Breastfeeding-Epigenetic Literature Review
- Decrease in LEP methylation could be one of the mechanisms by which breastfeeding contributes to protection against childhood obesity
- Because of its interaction with almost all neuropeptides that are involved in the regulation of energy balance and food intake,
- Leptin is important in the programming of metabolic pathways (Obermann-Borst, 2013, pg 3)

Breastfeeding-Epigenetic Literature Review
- Hunger Winter in the Netherlands during the winter of 1944
- Insulin Growth Factor (IGF) 2 / H19 gene
- Infants exposed to famine in utero and first trimester of pregnancy showed low birth weight compared with unexposed individuals
- Increased risk of obesity and cardiovascular disease as adults (Heijmans, 2008).

Breastfeeding-Epigenetic Literature Review
- Exposed to famine during the Dutch Hunger Winter had, six decades later, showed less DNA methylation of the imprinted Insulin Growth Factor (IGF) 2 / H19 gene
- Epigenetic marks as differences in methylation of the IGF2 / H19 affect the phenotypic expression associated with an increased risk of adult disease (Heijmans, 2008).

Breastfeeding-Epigenetic Literature Review
- Early catch up growth in infants born preterm
- Reduced fat mass at birth and who were formula fed
- Increase risk of cardio-metabolic disease and obesity in later life, (Barker, 2012).
- In utero or early postnatal development, short-term changes through environmental influences could permanently change organ development at a time of extreme vulnerability or “plasticity” (Koletzko, 2012).
Our Research - Purpose

- Our research examines the duration of breastfeeding and the phenotypic variation in growth in children enrolled in the Peri/Postnatal Epigenetic Twins Study (PETS).
- Potential epigenetic mechanisms for childhood obesity.
- Methylation of LEP and H19 genes.

Methods

- Cross-sectional data from a cohort at the 18 month visit (n=179) in the (PETS) was used to assess the relationship between duration of breastfeeding and infant size at 18 months of age.
- Epigenetic marks on the genes associated with controlling growth.

Inclusion criteria

- All participants that answered either
  - “less than 1 month”,
  - “1-3 months”
  - “4-6 months”
  were included in the analysis.
- Birth weight > 2000 grams

Exclusion criteria

- For the purposes of this BFG study, exclusion criteria included a birth weight below 2000 grams or a congenital disease affecting appetite, feeding or growth.

Breastfeeding cohort

<table>
<thead>
<tr>
<th>Duration of BF (n=179)</th>
<th>&lt; 1 month (Group D) (n=44)</th>
<th>1-3 months (Group C) (n=66)</th>
<th>4-6 months (Group B) (n=69)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24%</td>
<td>37%</td>
<td>39%</td>
</tr>
</tbody>
</table>
Statistical methods

- Means, ANOVA and t-test.
- Confounding variables were corrected for in the secondary linear analysis.
- Statistical significance was set at $P<0.05$.
- Statistical analysis was conducted using SAS version 9.3.

Consents

- Written consent was obtained at recruitment during the second trimester of pregnancy.
- All procedures were approved by the appropriate Australian ethics committees.
- The Institutional Review Board of Clemson University in Clemson, South Carolina approved this study.

Hypothesis

- Our hypothesis was the anthropometric measurements of the participants will be greater with shorter duration of breastfeeding.
- Epigenetic marks will be associated with genes identified with growth.

Anthropometric measurements

- Anthropometric measurements:
  - BMI
  - Ponderal index
  - Head circumference
  - Left arm circumference
  - Abdominal circumference
  - Tricep skinfold thickness
  - Subscapular skinfold thickness

Confounding variables

- Mother’s confounding variables
  - maternal age at delivery
  - education
  - smoking
  - alcohol
  - folate during the first trimester
  - maternal weight gain
  - gestational diabetes

- Child’s confounding variables
  - birth weight
  - gestational age
  - gender
  - zygosity
  - chorionicity
  - twin to twin transfusion syndrome
Linear statistical model

- A three factor linear model for each anthropometric measurement was developed that included terms:
  1) main effect of duration of breastfeeding category with the mother's ID category
  2) main effect of the confounding variable
  3) interaction of the confounding variable and duration of breastfeeding

Results - BMI

<table>
<thead>
<tr>
<th>Duration of breastfeeding (months)</th>
<th>Mean body mass index (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BF &lt; 1m</td>
<td>16.0 ± 0.5</td>
</tr>
<tr>
<td>BF 1-3m</td>
<td>16.5 ± 0.6</td>
</tr>
<tr>
<td>BF 4-6m</td>
<td>16.9 ± 0.7</td>
</tr>
</tbody>
</table>

Results - Arm Circumference

<table>
<thead>
<tr>
<th>Duration of breastfeeding (months)</th>
<th>Mean Left Arm Circumference (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BF &lt; 1m</td>
<td>15.8 ± 0.4</td>
</tr>
<tr>
<td>BF 1-3m</td>
<td>16.0 ± 0.5</td>
</tr>
<tr>
<td>BF 4-6m</td>
<td>16.2 ± 0.7</td>
</tr>
</tbody>
</table>

Results - Abdominal Circumference

<table>
<thead>
<tr>
<th>Duration of Breastfeeding</th>
<th>Mean Abdominal Circumference (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BF &lt; 1m (Group D)</td>
<td>47.0 ± 0.8</td>
</tr>
<tr>
<td>BF 1-3m (Group C)</td>
<td>46.8 ± 0.6</td>
</tr>
<tr>
<td>BF 4-6m (Group B)</td>
<td>46.5 ± 0.4</td>
</tr>
</tbody>
</table>

Results - Confounding Variables

- Three confounding variables modified the relationship between duration of breastfeeding and head circumference
  - Monozygosity
  - Monochorionicity
  - Female gender

Results - Summary

<table>
<thead>
<tr>
<th>Mean anthropometric measurements at 18 months</th>
<th>Duration of Breastfeeding Groups with Significance Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 1 month(D)/ 1-3 months (C) / 4-6 months(B)</td>
</tr>
<tr>
<td>Body mass index</td>
<td>- / - / 0.02</td>
</tr>
<tr>
<td>Ponderal index</td>
<td>- / - / -</td>
</tr>
<tr>
<td>Head circumference</td>
<td>- / - / -</td>
</tr>
<tr>
<td>Left arm circumference</td>
<td>- / 0.009 / 0.006</td>
</tr>
<tr>
<td>Abdominal circumference</td>
<td>- / - / 0.03</td>
</tr>
<tr>
<td>Tricep skinfold</td>
<td>- / - / -</td>
</tr>
<tr>
<td>Sub-scapular skinfold</td>
<td>- / - / -</td>
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</table>

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Methylation data

<table>
<thead>
<tr>
<th>Duration of breastfeeding</th>
<th>Methylation data at 18 months from DNA in saliva</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1 month (Group D) N=3</td>
<td>1057 twin 1 DZ 3006 twin 1 DZ 3006 twin 2 DZ</td>
</tr>
<tr>
<td>1 - 3 months (Group C) N=3</td>
<td>3014 twin 2 MZ 1058 twin 1 MZ 1058 twin 2 MZ</td>
</tr>
<tr>
<td>4 - 6 months (Group B) N=2</td>
<td>1072 twin 1 DZ 1072 twin 2 DZ</td>
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Methylation of Leptin promoter

<table>
<thead>
<tr>
<th>Array Number</th>
<th>CHROMOSOME</th>
<th>UCSC_RefGene_Name</th>
<th>Known regulatory regions</th>
</tr>
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<tbody>
<tr>
<td>cg03084214</td>
<td>7</td>
<td>LEP</td>
<td>leptin promoter</td>
</tr>
<tr>
<td>cg12782180</td>
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Methylation of the H19 promoter DMR

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<tr>
<td>cg24409677</td>
<td>11</td>
<td>H19</td>
<td>H19 promoter DMR</td>
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<tr>
<td>cg16303279</td>
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<td>cg02694715</td>
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</tr>
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<td>H19 promoter DMR</td>
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<td>cg01539474</td>
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Results - Summary

<table>
<thead>
<tr>
<th>UCSC_RefGene_Name</th>
<th>Known Regulatory Regions</th>
<th>Duration of Breastfeeding Groups with Significance Levels (p&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H19</td>
<td>H19 promoter DMR</td>
<td>0.023 0.019 0.009</td>
</tr>
<tr>
<td>H19</td>
<td>H19 promoter DMR</td>
<td>0.030 0.030 0.018</td>
</tr>
</tbody>
</table>

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Results – Epigenetic Pattern vs Phenotype

- Significant associations between duration of breastfeeding:
  - BMI
  - Arm circumference
  - Abdominal circumference

- Breastfeeding for less than 4 months was associated with a larger arm circumference, a larger abdominal circumference and a higher BMI.

Results – Discussion on epigenetic patterns

- Significant associations between duration of breastfeeding:
  - Methylation of two adjacent H19 promoters on chromosome 11.
  - Breastfeeding for less than 4 months was associated with hypermethylation of the H19 promoters in the PETS.
  - Breastfeeding for longer than 4 months was associated with hypomethylation of both H19 promoters.

Results – Discussion on phenotype

- May be a relationship between the hypomethylation on two adjacent H19 promoters and the phenotypic variation of a smaller BMI, arm and abdominal circumference associated with breastfeeding for more than 4 months.
- There were no significant differences in methylation on the Leptin gene between the three groups of varying duration of breastfeeding.
- Due to the limited amount of methylation data in the breastfeeding cohort a definitive relationship can not be concluded.
### Results - Discussion

- Many conflicting studies analysing breastfeeding duration with childhood growth, but first to study:
  - a cohort of twins
  - comprehensive list of prenatal life factors
  - including sharing of the prenatal and postnatal environment
  - in combination with access to DNA methylation data

### Limitations

- Limitations include:
  - the small number of participants in the breastfeeding cohort (n=179)
  - Limited amount of methylation data (n=8)
  - not accounting for the use of nutritional supplements
  - stringent exclusion criteria reduced the sample size
  - twin data base limits generalizability

### Strengths

- Strengths of our study are
  - variables accounted for in the prenatal and postnatal environmental
  - access to DNA methylation data.
  - mother’s numerical identification numbers were paired with the twins to account for the influence of sharing the same in-utero and home environment as a confounding variable

### Strengths

- linear model strengthens the conclusions of the study by accounting for the environmental influences.
  - standardized visit at the 18 months after birth, thereby, minimized recall bias
  - accuracy and internal validity of the anthropometric measurements
  - cross sectional, longitudinal database.

### Conclusions

- Supplementing with non-breast milk before 4 months of age was associated with increased BMI, arm circumference and abdominal circumference at 18 month months of age.
- Breastfeeding for 4-6 months appeared to protect against the risk of obesity for the 18 month old children enrolled in the PETS.
- Hypomethylation of H19 promoter may be protective against childhood obesity.

### Conclusions

- Mean BMI decreased from 85% to 65% when infants were BF for 4-6 months compared to infants BF for 1-3 months.
- Hypomethylation of the H19 promoter DMR was associated with a lower BMI, smaller arm and abdominal circumference in the infants breastfed for 4-6 months in the PETS.
Implications
• Understanding the interactions of diet and nutrition with our epigenome allow healthcare providers to tailor education and interventions focused on early life nutritional habits to help prevent obesity.

Tailoring education on nutritional habits
• “Human breastmilk...is the most specific personalized medicine, given at the time when gene expression is being fine-tuned for life. This is an opportunity for health imprinting that should not be missed” (Victoria, 2016 pg. 486)

Stressing the epigenetic benefits
• Reduction in Obesity: 26% reduction in the odds of overweight of obesity* (Horta, 2015)
• Reduction in Type 2 diabetes: 35% reduction (95% CI 14–51) (Victoria, 2016)
• Higher IQ: 3-4 intelligence quotient (IQ) points (Horta, 2015) and 7 IQ points at 6.5 years of age (Kramer, 2008)
• Lower Neonatal Necrotizing Enterocolitis (NEC): 77% reduction risk in preterm infants compared to formula (AAP, 2012)
• Less maternal breast cancer: (Collaborative Group on Hormonal Factors in Breast Cancer, 2002).

Future Research
• Increasing the amount of methylation data in the PETS breastfeeding cohort to further investigate epigenetic mechanisms in early feeding patterns for later onset obesity.
• Investigating the unique hormones, peptides, stem cells, metabolites and neurotransmitters in breast milk may provide an epigenetic mechanism in early life contributing to obesity.

Future Research
• We recently identified a novel candidate gene which has 5 probes within 1 kb of each other that appeared to have a strong effect size (between 13% to 35%) (P = .03) in the statistical analysis.
• This gene encodes a component of a signaling pathway that regulates cell growth in response to nutrient and insulin levels.
• We have funding to analyze the methylation for the remaining 171 participants at the site of this novel gene this summer at the Murdoch Childrens Research Institute in Australia. So stay tuned......

Acknowledgments
• Drs. Jeffrey Craig & Richard Saffery
• Peri/Prenatal Epigenetics Twins Study (PETS)
• School of Nursing and the Department of Genetics and Biochemistry
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References


