Comparative Glycomics in Mother's Milk of Premature and Term Infants
Across In Vivo Digestion

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BACKGROUND

INFANT DIGESTION

The premature infant may not be fully competent to digest intact breast milk. Compared to the gut of term infants, the premature gut is structurally, functionally and developmentally immature. The premature gut produces less gastric acid and hydrolytic enzymes and thus breaks down the complex polymers of milk, e.g. protein less effectively. The main gastric proteolytic enzyme, pepsin, is at much lower levels in premature infants than in term infants. Reports on enzyme levels in the intestinal tract are conflicting for proteolytic enzymes, but some suggest that the levels are much lower in the premature infant. The consequences of this prematurity are myriad and include:

- INACTIVE PEPTIDES
  Many milk peptides (protein fragments) have shown biological effects in the gastrointestinal tract (GIT) and the blood.
- Loss digestion in preterm infants leads to different released peptides and a different array of biological effects.
- The forms of these peptides as digested in the infant stomach have never been determined.

GLYCOYSIS

- Glycosylation of proteins has been shown to have structural implications and functional effects
- Humans do not have the genetic capabilities to break glycosyl bonds.
- It is not known if milk protein glycans are broken down in infant GIT.
- It is not known if glycosylation affects digestion in full or preterm infants.

HYPOTHESIS

- Premature infants produce a digestive profile from human milk that is distinct from full term infants.

OBJECTIVE

1. Determine composition of human milk proteins n-linked glycans of pooled milk
2. Determine glycan composition of milk at 1, 2 and 3 hrs post gastric digestion

STUDY DESIGN

Subjects: Term infants (37-41 weeks) and premature infants (25-29 weeks) in the Neonatal Intensive Care Unit of UC Davis Medical Center

Sampling:
- Removed by suction from oro-gastro-feeding tubes (already in place due to medical necessity some of which may affect the digestion process)
- All infants are described and followed
- Volume: 2 ml
- Samples: Undigested breast milk from each mother and stomach contents at 1, 2 and 3 hours post-digestion.

DATA ANALYSIS

- Data collected for each infant/digest/milk sample includes:
  - Location of collection in GI tract
  - Time point (by hour) of sampling
  - Sample collection date
  - Infant day of life at sampling
  - Biochemistry
  - Infant characteristics
  - Sample comparisons and statistical analyses

MATERIALS AND METHODS

- n-linked glycan extraction and analysis
  - Start with 500 µl sample (human milk or stomach contents)
  - Centrifuge 30 min, to remove cream
  - Precipitate with ethanol to remove human milk oligosaccharides (HMO)
  - Solubilize and denature
  - Divide each sample into two 200 µl fractions
  - Add 2 µl Popelin N-Glycoloylase (PNGase F) to one of each of the divided samples
  - PNGase F cleaves N-linked glycans from protein
  - Place in microwave reactor for 10 minutes at 20 watts, 60 C
  - Centrifuge 30 min, to remove cream
  - Glycosidase F (PNGase F) to one of each divided sample
  - Glycosidase F removes carbohydrate from glycoprotein
  - Graphitized carbon chromatography (GCC) cartridges for glycan clean-up with Gilson Automatic Liquid Handling Robot
  - Elute with 10%, 20%, and 40% acetonitrile (ACN)
  - Analyze with Matrix-Assisted Laser Desorption/Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (MALDI-FTICR MS) in positive mode

RESULTS

- Individual Spectra Results (Z4.6 PTY 10):
  - Comparison of a single sample divided into one with and one without PNGase F allows for simple identification of human milk glycans (HMG).
  - All peaks matched to carbohydrate compositions present in the sample with PNGase F and not present in the sample without are most likely HMG.
  - HMG possibilities then trimmed down based on biological knowledge of what is actually possible (i.e. must have 2 hex, 3 hex to be glycan)
  - Figure (what #?): Mass spectra of term milk, with no glycan release, eluted with 10% ACN

- Single Experiment Results
  - To determine whether small glycopeptides exist in intact human milk, sample was applied to 10 kDa molecular weight cut-off membrane spin column. Peptides and glycopeptides smaller than 10 kDa flowed through the membrane, while those greater than 10 kDa were retained. The fractions were then analyzed for HMG content and composition after PNGase F release

- Table #:
<table>
<thead>
<tr>
<th>Sample</th>
<th>PNGase F</th>
<th>HMG Presence</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>yes</td>
<td>present</td>
</tr>
<tr>
<td>25</td>
<td>no</td>
<td>not present</td>
</tr>
</tbody>
</table>

- More HMG found in >10 kDa fraction, but some found in <10 kDa fraction
- Finding HMG in <10 kDa fraction suggests presence of small glycopeptides in intact human milk

Overall Experimental Results

- Figure #: 2D DIGE (2D-DIGE)
  - For an overall view of digestion differences between protein and term infants, 2D DIGE were run with a set of term and preterm infants matched for days post-partum and fed donor milk.
  - Proteins and term infant proteins were dried with green and red fluorescent dyes, respectively

- Figure #: 2D DIGE images of isolated protein from gastric samples of 35 day old premature infant fed donor milk at 1 and 2 hours post-inigestion. Image shows the overlap of the two samples individually fluorescent.

CONCLUSIONS

- Analytical platform combined with custom computational programming is capable of capturing the complexity of digestive contents of premature and full term infants.
- First generation comparisons tell a compelling story of differences between premature and full term infants.
- Major challenges now are to annotate these changes and identify critical needs for assisting premature infants

IN PROGRESS

- Establishing the boundaries of variation in composition and hydrolytic activity across infants
- Allowing retrospective analysis of HMG changes over time of digestion in stomach and intestinal tract of term and preterm infant

FUTURE STEPS

- Determine glycopeptide profile of infant digestion of human milk
- Identify specific peptides whose lack of activity constitute a clinically measureable liability to premature infants

ACKNOWLEGEMENTS

Two-dimensional Difference In Gel Electrophoresis (2D-DIGE) experiments

- Protein isolation
- Insert method
- Gel
- Run by ____ company