Necrotizing Enterocolitis: an Update

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"Classic" NEC


Agenda

- Background
- Different forms of "NEC"
- Pathogenesis: "Classic NEC"
- Can we develop predictive and/or diagnostic biomarkers?
Necrotizing Enterocolitis: Scope of the Problem

- In US and Canada, it affects approximately 7% of babies weighing between 500-1500 grams (approximately 20-30% die).
- Approximates the number of children fewer than 15 years of age who die of leukemia or meningitis.
- Survivors may be left with significant sequelae.
- Very costly.
- Scares neonatologists!

More than 1 Disease: What’s been called “NEC”?

- Term infants
- Preterm infants with isolated intestinal perforations.
- Preterm infants—“Classic” NEC

NEC in Term Infants vs. Preterms

- In term infants: Greater association with:
  - Congenital Heart Disease.
  - “Differences in initial severity, range of age at diagnosis, and outcomes between subjects with necrotizing enterocolitis with and without cardiac disease suggest that necrotizing enterocolitis in the cardiac patient is a distinct disease process and should be labeled cardiogenic necrotizing enterocolitis” Pickard, et al Pediatrics, 2009
Right after birth 12 hours later

Isolated Non-NEC Perforations

- Occurs early.
- Not related to feedings (except for possibly the lack of feedings).
Bells is Broken

- Stage 1 - Too non-specific and the term should not be used.
- Stage 3 - Could signify intestinal necrosis or Spontaneous Intestinal Perforation

What Causes NEC?

Rat model of “NEC”.
The 'Myth' of Asphyxia and Hypoxia-Ischemia as Primary Causes of Necrotizing Enterocolitis

Commentary to Green et al.: Insulin-Like Growth Factor Attenuates Apoptosis and Macrophage Damage in Hypoxia/Reoxygenation-Induced Intestinal Injury (Biol Neonate 2006;91:91–96)

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Neu, J. Acta Paediatrica, 2005
94 (Suppl 449): 100-105


Innate Immune System Immaturities:
Extrinsic Barriers
Gastric Acid Secretion

H-2 Blocker Therapy and Higher Incidence of NEC

- NICHD Neonatal Network
- Case Control study of Bell Stage II or greater
- 11,072 infants (BW 401-1500 gm), 7.1% developed NEC.
- Antecedent H-2 blocker therapy resulted in higher incidence of NEC (P<0.001).


Gastric Acid Inhibition

Ranitidine is Associated With Infections, Necrotizing Enterocolitis, and Fatal Outcome in Newborns

Pediatrics, 2012, 129. e-40-45
NEC: Relation to Inflammatory Mediators

- Increased plasma pro-inflammatory cytokine (IL-8, IL1, IL-6, TNF-α) concentrations.
- Increased platelet activating factor.

The Gut is the Motor that Drives Systemic Inflammation and Multiple Organ Dysfunction!

Is the Immature Intestine More Prone to Inflammation than the Adult?

IL-8 secretion (A) and IL-8 mRNA induction (B) in fetal and infant organ culture preparations in response to LPS (50 µg/ml) or media alone as control

(Nanthakumar et al., PNAS, 2000).
The Tight Junction

Barrier Function: loss of Epithelial Integrity

Factors Affecting TJ Function

- Enteral nutrient deprivation (e.g., TPN).
- Stress (trauma, burns, surgery and psychological).
- Infection.
- Cytokines, vascular endothelial growth factor.
- Drugs (e.g., Indomethacin and glucocorticoids).
- Microbial Ecology
The Human Microbiome Project

Goal: identify and characterize the microorganisms which are found in association with both healthy and diseased humans (their microbial flora).

Commensal Microbes: Beneficial Effects for the Host

- Nutrient metabolism
- Tissue development
- Resistance to colonization with pathogens
- Maintenance of intestinal "homeostasis"
- Development of the Brain?
Regional Differences

Conventional vs. Germ Free Mice

“TOLL” Proteins
“Toll Receptors”

Lessons

- Low grade stimulation ("tickling") of toll receptors can prevent high grade inflammation and intestinal damage.
- What are the mechanisms?

Results
Effects of the cell components Flagellin, LTA and LPS on IL-8 production.
**Microbiota Study of Babies with NEC at UF**

- Preterm infants with birth weight less than 1250 grams and gestational age <32 weeks were enrolled from three University of Florida affiliated hospitals.
- Stools collected from birth, frozen and stored.
- Stools from babies who developed NEC taken for analysis.
- Control infants were selected and matched to NEC case infants using gestational age, birth weight, birth center, date of birth (+/- 2 months), and predominant enteral nutrient (breast milk vs. formula).

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**Table 1. Characteristics of study subjects.**

<table>
<thead>
<tr>
<th>Infant</th>
<th>Gender</th>
<th>Race/Ethnicity</th>
<th>Birth Weight</th>
<th>Gestational Age</th>
<th>Enteral Nutrient</th>
<th>NEC</th>
<th>Grad</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant A</td>
<td>F</td>
<td>White</td>
<td>1500 grams</td>
<td>28 weeks</td>
<td>Breast milk</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Infant B</td>
<td>M</td>
<td>White</td>
<td>1350 grams</td>
<td>27 weeks</td>
<td>Breast milk</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Infant C</td>
<td>F</td>
<td>Black</td>
<td>1400 grams</td>
<td>30 weeks</td>
<td>Formula</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Notes: Gestational age in weeks, Birth weight at birth in g, Gender (M, female; F, male), Race/Ethnicity (White, Black, Hispanic), Enteral Nutrient (Breast milk, Formula), NEC (Yes, No), Grad (Yes, No).
Fecal Microbiota: NEC

Mai V, Young C. PLOS One, May 2011

1 week before

Within 72 hrs of diagnosis

2 weeks before

1 week before

Less than 1 week before NEC
UniFrac metric analysis: grouping of the UF Jacksonville’s samples together separately from UF-Gainesville samples.


TOP TEN LIST OF NICU MEDICATIONS

<table>
<thead>
<tr>
<th>Medication Name</th>
<th>Frequency, A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>196,799</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>117,180</td>
</tr>
<tr>
<td>Fentanyl Sulfate</td>
<td>90,152</td>
</tr>
<tr>
<td>Intravenous (methylprednisolone)</td>
<td>64,129</td>
</tr>
<tr>
<td>Cilostazol</td>
<td>55,465</td>
</tr>
<tr>
<td>Caffeine citrate</td>
<td>48,874</td>
</tr>
<tr>
<td>Furosemide</td>
<td>47,376</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>44,218</td>
</tr>
<tr>
<td>Benadel (Cephalixin)</td>
<td>36,410</td>
</tr>
<tr>
<td>Moraabatamide</td>
<td>27,541</td>
</tr>
</tbody>
</table>
Neonatal Antibiotic Treatment and Gastrointestinal Tract Development?

• 100 mg/kg/d Clamoxyl compared with saline control.
• All bacteria were significantly reduced especially Lactobacillus, mainly in colon.
• Affymetrix gene microarrays performed.
• 10-30% of the genes undergoing maturational changes showed modulation by the antibiotic so that their normal pattern of maturation was either accelerated or slowed down.
• MHC genes markedly affected—required for tolerization to luminal antigens.


Odds ratio of NEC with increased days on antibiotics


“Classic” NEC: Diagnostic Biomarkers

Why are new biomarkers needed for NEC?

• Current diagnosis based on history, physical exam, radiographic criteria detects the disease after it’s occurrence when it may already be too late.
• Diagnostic biomarkers would enhance our diagnostic capabilities.
• Predictive biomarkers would tell us which infants are at highest risk so that we could provide targeted prevention.
**CRP, WBC and PLATELETS**

Thuijls, et al. Annals of Surgery, 251 (6), June 2010

- PLATELETS
- C-REACTIVE PROTEIN
- WHITE BLOOD CELL

**Claudin**

Thuijls, et al. Annals of Surgery, 251 (6), June 2010

- Released by intestinal neutrophils with intestinal inflammatory damage and can be used as a marker for gut wall inflammation.
- It is resistant to enzymatic degradation, and can be easily measured in feces.

**Calprotectin**

Thuijls, et al. Annals of Surgery, 251 (6), June 2010

- Released by intestinal neutrophils with intestinal inflammatory damage and can be used as a marker for gut wall inflammation.
- It is resistant to enzymatic degradation, and can be easily measured in feces.
INTESTINAL FATTY ACID BINDING PROTEIN (I-FABP)
Thuijls, et al. Annals of Surgery, 251 (6), June 2010

- Small, water-soluble protein limited to mature enterocytes of small and large intestine.
- Released into circulation when cell membrane integrity is compromised.
- Can be measured in urine.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cutoff Value</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>LR+</th>
<th>LR-</th>
<th>AUC (95%CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-FABP</td>
<td>2.25 pg/mmol creatinine</td>
<td>0.93</td>
<td>0.90</td>
<td>9.3</td>
<td>0.08</td>
<td>0.98 (0.94-1.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Claudin-3</td>
<td>800.8 INT</td>
<td>0.71</td>
<td>0.81</td>
<td>3.74</td>
<td>0.36</td>
<td>0.76 (0.59-0.94)</td>
<td>0.016</td>
</tr>
<tr>
<td>Calprotectin</td>
<td>286.2 microgram/gram feces</td>
<td>0.86</td>
<td>0.93</td>
<td>12.29</td>
<td>0.15</td>
<td>0.94 (0.85-1.0)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

NEC versus Non NEC Differentiation
Thuijls, et al. Annals of Surgery, 251 (6), June 2010

ApoSS for Sepsis/NEC
Ng, PC et al., J Clin Invest. 2010 August 2; 120(8): 2989–3000

Figure 4
A large strategy for classifying cases suspected for late-onset sepsis/NEC on the basis of ApoSS scores on day 6 and day 1.
Inter-Alpha Inhibitor Protein

PREDICTIVE BIOMARKERS: Who is at greatest risk?

Buccal swab or Salivary analyses: Predictive Biomarkers
Salivary Secretor Phenotypes

<table>
<thead>
<tr>
<th>Secretor phenotypes</th>
<th>Control</th>
<th>Case</th>
<th>Odds Ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>11</td>
<td>9</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Normal</td>
<td>14</td>
<td>10</td>
<td>3.4 (2.1-5.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>High</td>
<td>23</td>
<td>15</td>
<td>3.4 (2.1-5.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Does not secret</td>
<td>20</td>
<td>14</td>
<td>1.00 (0.6-1.7)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Source: Information was not determined in 2 people having a each allele single, including 2 cases and 20 controls. The P value determination will be based on the data for the remaining cases and controls.


Biomarker Discovery and Validation

Protein Spot Identification
<table>
<thead>
<tr>
<th>No.</th>
<th>Protein Name</th>
<th>Fold</th>
<th>p-value</th>
<th>mass (kDa/pI)</th>
<th>IPI number</th>
<th>score</th>
<th>coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Interleukin-1 receptor antagonist (IL-1RA)</td>
<td>-3.15</td>
<td>0.029</td>
<td>16.23/5.01</td>
<td>IPI00174541</td>
<td>256</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>Peroxiredoxin-1</td>
<td>-2.19</td>
<td>0.01</td>
<td>18.09/5.05</td>
<td>IPI00000874</td>
<td>274</td>
<td>39</td>
</tr>
<tr>
<td>3</td>
<td>Isoform 1 of Alpha-1-antitrypsin</td>
<td>3.18</td>
<td>0.045</td>
<td>72.77/5.53</td>
<td>IPI00553177</td>
<td>117</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>Clusterin isoform (apolipoprotein J)</td>
<td>-2.16</td>
<td>0.0087</td>
<td>65.50/7.62</td>
<td>IPI00291262</td>
<td>140</td>
<td>8.91</td>
</tr>
<tr>
<td>5</td>
<td>Proteosome subunit alpha type 2</td>
<td>1.18</td>
<td>0.047</td>
<td>26.04/5.05</td>
<td>IPI00219622</td>
<td>85</td>
<td>13.2</td>
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<tr>
<td>6</td>
<td>Gelsolin (isoform 2-cytoplasmic)</td>
<td>1.78</td>
<td>0.019</td>
<td>81.26/5.61</td>
<td>IPI00646773</td>
<td>698</td>
<td>22</td>
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<tr>
<td>7</td>
<td>Cleaved Peroxisomal multifunctional enzyme type 2</td>
<td>-3.61</td>
<td>0.0016</td>
<td>38.60/8.78</td>
<td>IPI00019912</td>
<td>298</td>
<td>9.92</td>
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<tr>
<td>8</td>
<td>Phosphatidylethanolamine-binding protein 1</td>
<td>4.18</td>
<td>0.004</td>
<td>23.53/8.59</td>
<td>IPI00219446</td>
<td>243</td>
<td>26</td>
</tr>
<tr>
<td>9</td>
<td>Alpha-2-glycoprotein 1, zinc precursor</td>
<td>-2.24</td>
<td>0.0062</td>
<td>38.70/5.41</td>
<td>IPI00166729</td>
<td>112</td>
<td>26</td>
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<tr>
<td>10</td>
<td>Polymeric immunoglobulin receptor</td>
<td>2.08</td>
<td>0.0026</td>
<td>86.63/5.24</td>
<td>IPI00004573</td>
<td>238</td>
<td>7.3</td>
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<td>11</td>
<td>(3R)-hydroxyacyl-Co A dehydrogenase)</td>
<td></td>
<td></td>
<td></td>
<td>IPI00004798</td>
<td>297</td>
<td>12</td>
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<tr>
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<td>Alpha-2-glycoprotein 1, zinc precursor</td>
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<td>0.0062</td>
<td>38.70/5.41</td>
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<td>7.3</td>
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<td></td>
<td></td>
<td>IPI00004798</td>
<td>297</td>
<td>12</td>
</tr>
</tbody>
</table>

### Pathway Analysis

![Pathway Diagram](image)

### Buccal IL-1 RA: First Week

![Graph](image)
Take Home Messages

• “NEC” is more than one disease.
• Pathogenesis of “classic NEC” is multifactorial, but some factors (e.g., GI microbial ecology) may be more important than others.
• Predictive and diagnostic biomarkers are needed for targeted prevention and therapy.