Novel biomarkers for early and accurate detection of a fatal gut inflammatory disease in preemie babies

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Abstract

Necrotizing enterocolitis (NEC) is an inflammatory disease that primarily affects the intestinal tract of premature and low birthweight infants. It is one of the most common complications that occur with prematurity, which also results in high morbidity and mortality due to unchecked pathogenic bacterial growth. The median time between death and x-ray diagnosis is 1 day and, currently, there are no reliable molecular methods to predict the onset of NEC in infants. Association of intestinal alkaline phosphatase (iAP) with moderate and severe forms of the disease suggested that iAP can be a diagnostic tool that is accurate and specific for NEC. This study aims to determine the potential of iAP as a predictive biomarker for NEC. Fecal samples were obtained prospectively from premature infants admitted to neonatal intensive care units at four hospitals (Children's Hospital New Orleans, Woman's Hospital Baton Rouge, Touro Infirmary, and Washington University of St. Louis Medical School). More than 100 clarified stool samples from case patients were compared to 200+ age-matched control samples. Biospecimens were analyzed for iAP abundance, iAP enzyme activity, and total fecal protein concentration. Analyses of age-matched NEC and control samples show increased iAP abundance and decreased enzymatic activity directly correlate with NEC diagnosis as early as 3 days before x-ray and a hazard ratio of 6. These findings suggest iAP shows promise as a marker for early NEC detection in asymptomatic infants. If confirmed with a larger study, an iAP biomarker could allow physicians to identify at-risk infants that require medical intervention and allow them to personalize treatment to slow or even stop disease progression altogether.

We prospectively enrolled 259 premature infants at four hospitals from 2015-2021

Table 1. Clinical characteristics of NEC cohorts. Data are median *(IQR)* or n. Using appropriate method (ANOVA, Kruskal-Wallis test, Fisher's exact test, or Student's t-test) to compare difference among three groups and an α value of 0.05, *p*-values ≤ α support that there are statistically significant differences between the three infant populations. Statistically meaningful *p*-values are shown in bold.

Clinical measure	advanced NEC	early NEC	non-NEC controls	*p*-value
Birthweight (g)	855 *(700-1380)*	940 *(790-1190)*	1100 *(845-1380)*	0.28
Gestational age (wk)	27.6 *(24.7-31.1)*	28.0 *(26.0-29.4)*	28.7 *(26.4-31.6)*	0.48
Sex				0.39
male	13 *(52%)*	12 *(63%)*	42 *(46%)*	
female	12 *(48%)*	17 *(37%)*	49 *(54%)*	
Race/ethnicity				0.08
African American	10 *(40%)*	14 *(74%)*	63 *(69%)*	
Caucasian	13 *(52%)*	5 *(26%)*	24 *(26%)*	
Hispanic	2 *(8%)*	0 *(0%)*	2 *(2%)*	
Other	0 *(0%)*	0 *(0%)*	2 *(2%)*	
Age of first NEC episode				
PCA (wk)	33.9 *(31-35.7)*	29.4 *(28.4-30.9)*	N/A	**0.02**
weight (g)	1620 *(1110-2050)*	1015 *(860-1377)*	N/A	**0.0006**
# sepsis co-morbidity	9 *(35%)*	4 *(21%)*	13 *(14%)*	0.24
Total # blood transfusions	5 *(2-11)*	5 *(1-6)*	0 *(0-3)*	**<0.0001**
Exposure to human milk				0.31
0%	4 *(16%)*	3 *(16%)*	8 *(9%)*	
<10%	4 *(16%)*	0 *(0%)*	10 *(11%)*	
10-50%	4 *(16%)*	2 *(10%)*	19 *(21%)*	
≥51%	13 *(52%)*	14 *(74%)*	54 *(59%)*	

- Thirty-eight infants were diagnosed with advanced NEC (Bell stage II/III), 28 with early NEC (stage I), and 193 were non-NEC infants. Twenty-six infants had sepsis and 15 had non-GI infection.
- There were no statistically significant correlations with birthweight, gestational age, race, Apgar score, or human milk exposure between development of NEC with *pneumatosis intestinalis*, suspicion of NEC, and control patients.
- The only meaningful clinical association with NEC was post conceptual age, supporting the model that the disease develops after birth.
- Over 2,500 stool specimens were obtained, and 4,200 assays were completed on 600 of them. We compiled 5,400 demographic and general disease course characteristics. Lastly, we collated 163,200 entries regarding their hospital stay.

References

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Introduction

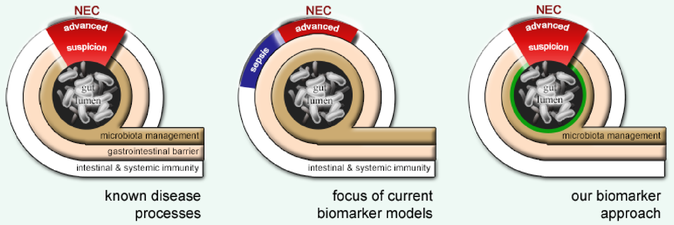

Figure 1. The identity of key players in gut lumen homeostasis that give rise to NEC age-dependence is unknown[1]. ***(A)*** NEC is correlated with several physiological and structural processes in the gut (top panel) and is most prevalent between 26-35 wks postconceptual age (bottom panel). ***(B)*** Current research effort to develop a NEC biomarker has focused on proteins in immunity cascades, which invariably are not specific for NEC, and in dysbiosis of the microbiome. ***(C)*** Our approach focused on host proteins involved in microbiota management.

- Neonatal necrotizing enterocolitis (NEC) is a gastro-developmental disorder[1].
- Currently, diagnostic tools are limited. X-ray determination of pneumatosis intestinalis and/or portal air is used for diagnosis of advanced disease and lacks sensitivity (as low as 44%)[2].
- Proteins involved in intestinal and systemic inflammation are not specific markers for NEC; detection of inflammation biomarkers signal late stages of disease process and inflammatory damage[3,4].
- Although structural features of the gastrointestinal tract are largely in place at birth, candidate links to NEC are proteins in the microvilli that are responsible for functional maturation and adaptation to mutualistic microorganisms, when the infant in the extra-uterine environment[5].
- Our hypothesis is that **intestinal alkaline phosphatase (iAP),** involved in microbiota management, can be used to detect NEC earlier and allow for disease reversibility.

iAP measures identified advanced disease and early disease not observable by x-ray

- Cross-sectional analysis showed that high iAP content shed in gut lumen is always associated with NEC and has no correlation with sepsis or other non-GI infections.
- The absolute level of iAP activity was always lower for all infants diagnosed or suspected of NEC.

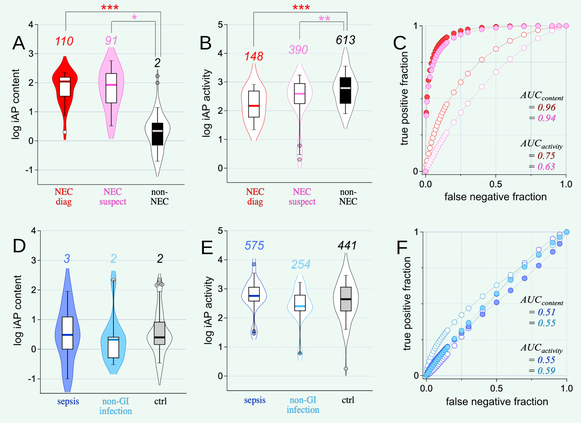

Figure 2. High iAP content and low iAP activity are linked with advanced stages of NEC and NEC suspicion[1]. Medians of relative iAP content ***(A)*** and for iAP activity ***(B)*** are shown for samples collected in the clinical window of NEC diagnosis (red horizontal line) and of NEC suspicion (pink horizontal line). Samples from non-NEC patients (black), at the average post-conceptual age for NEC diagnosis and suspicion, are also shown. Turkey IQR (boxes) were used to define outliers (circles) as hinge ± 1.5xIQR; there were no far outliers. Whiskers on the box plots are marked the 9th and 91st percentiles. Box plot is superimposed on a violin plot (solid color), which uses a kernel density estimate, and shows that the data distribution is continuous and not bimodal. Number of asterisks denote ten-fold differences in *p*-value, which was determined by 2-sided t-test and with unequal variance. In *(A)*, *p*-value for *** and for * is 0.0003 and 0.0124, respectively; in *(B)*, *p*-value for *** for ** is 0.0002 and 0.0064, respectively. ***(C)*** Receiver operator curves of the iAP content in samples collected during NEC diagnosis (filled red circles) and NEC suspicion (filled pink circles). Also shown are ROC curves for iAP activity in samples collected while infant was diagnosed with NEC (open red circles) or was suspected of having NEC (open pink circles). Medians of relative iAP content ***(D)*** and for iAP activity ***(E)*** are shown for samples collected during sepsis (dark blue) or other non-GI infection (light blue). Samples from control patients (black), at the average post-conceptual age for sepsis and non-GI infections, are also shown. ***(F)*** ROC curves of the iAP content (filled symbols) and iAP activity (open symbols) in samples collected in the clinical window of sepsis (dark blue) and other non-GI infections (light blue). Also shown are ROC curves N=14-91.

Multiplexed measures increased hazard ratio and earlier disease detection in asymptomatic infants

- Increased levels of iAP in stool occurs on average 3.3 days earlier than x-ray diagnosis of NEC.
- If iAP abundance and iAP activity are multiplexed, it increases the average number of days that a patient with NEC can be diagnosed in advance of x-ray to 4.9 days.
- An asymptomatic patient with elevated iAP abundance has a 6-fold higher likelihood of developing NEC while a patient with an elevated multiplex score is 19 times more likely to develop NEC.

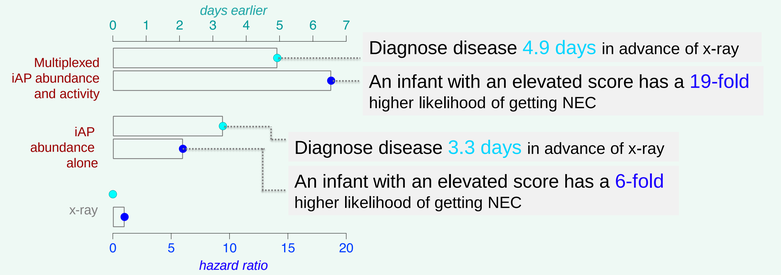

Figure 3. Samples collected longitudinally from 91 patients were analyzed to determine the likelihood a patient would develop NEC prior to x-ray diagnosis. IAP abundance alone and iAP abundance combined with iAP activity were assayed. The average number of days prior to x-ray diagnosis that a patient's stool had elevated amounts iAP compared to controls was determined. The number of days prior to x-ray diagnosis that a patient had an elevated abundance of iAP with significantly reduced iAP activity was also compared to controls. A hazard ratio was calculated to determine the fold likelihood that iAP abundance alone or multiplexed iAP abundance and activity could be used to indicate the likelihood of disease.

Conclusions

- Both iAP abundance alone and a multiplexed iAP score are promising candidates for early diagnoses of preterm necrotizing enterocolitis
- Several different types of opportunities would become available for clinical end users if a simple method for early and more accurate NEC diagnosis was available.

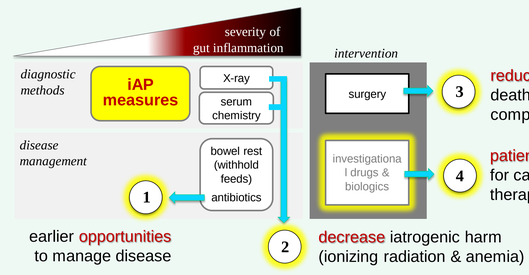

Figure 4. Potential patient benefits and clinical opportunities from earlier and more accurate diagnosis of NEC. Preemie standard of care requires new methods to monitor NEC disease. The gold standard for diagnosis is x-ray and identifies only 44%[2] of advanced NEC cases. Disease management choices in mid-to-advanced NEC are shown. Numbered circles, highlighted with yellow, mark potential changes in clinical workflow and outcomes from use of our proposed biomarker. Dark and light blue arrows show immediate benefits and clinical advances for patients, respectively.

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