Urine Protein Biomarkers for the Diagnosis and Prognosis of Necrotizing Enterocolitis in Infants

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Objectives To test the hypothesis that an exploratory proteomics analysis of urine proteins with subsequent development of validated urine biomarker panels would produce molecular classifiers for both the diagnosis and prognosis of infants with necrotizing enterocolitis (NEC).

Study design Urine samples were collected from 119 premature infants (85 NEC, 17 sepsis, 17 control) at the time of initial clinical concern for disease. The urine from 59 infants was used for candidate biomarker discovery by liquid chromatography/mass spectrometry. The remaining 60 samples were subject to enzyme-linked immunosorbent assay for quantitative biomarker validation.

Results A panel of 7 biomarkers (alpha-2-macroglobulin-like protein 1, cluster of differentiation protein 14, cystatin 3, fibrinogen alpha chain, pigment epithelium-derived factor, retinol binding protein 4, and vasolin) was identified by liquid chromatography/mass spectrometry and subsequently validated by enzyme-linked immunosorbent assay. These proteins were consistently found to be either up- or down-regulated depending on the presence, absence, or severity of disease. Biomarker panel validation resulted in a receiver-operator characteristic area under the curve of 98.2% for NEC vs sepsis and an area under the curve of 98.4% for medical NEC vs surgical NEC.

Conclusions We identified 7 urine proteins capable of providing highly accurate diagnostic and prognostic information for infants with suspected NEC. This work represents a novel approach to improving the efficiency with which we diagnose early NEC and identify those at risk for developing severe, or surgical, disease. *(J Pediatr 2014;164:607-12)*.

The underlying etiology of necrotizing enterocolitis (NEC) remains poorly understood but is thought to be multifactorial, involving factors inherent to the premature neonate and its environment. Specific features believed to be involved in the development of NEC include an underdeveloped gastrointestinal mucosal barrier, immature innate and humoral immunity, uncoordinated intestinal peristalsis, and pathogenic bacterial overgrowth.[1] Despite many advances in neonatal intensive care, NEC continues to be a major source of morbidity and mortality in preterm infants. It is diagnosed in 1%-5% of all patients in the neonatal intensive care unit, with an incidence of up to 15% reported in infants weighing less than 1500 g.[2,3]

NEC occurs across a spectrum of severity from a mild form that resolves with antibiotics and cessation of feedings (medical NEC) to a progressive form that leads to intestinal perforation, peritonitis, and potentially death (surgical NEC).[4] Approximately 20%-40% of all infants diagnosed with NEC eventually require surgery.[5] Although Bell's classification scheme, first introduced in 1978,[6] is useful in guiding initial treatment decisions, it does not serve as a prognostic instrument of disease progression.

Many previous attempts have been made to identify biologic markers for the early detection of NEC. Breath hydrogen levels, genomic analyses, targeted inflammatory marker detection, and fecal microbiota profiling have all shown initial promise as predictors of high-risk populations but have achieved limited clinical success for a variety of reasons.[7-15] In the current study, we used an unbiased exploratory proteomics approach to define a urine protein biomarker panel with the ability to enable both timely diagnosis and accurate prognosis for infants with presumed NEC.

A2ML1	Alpha-2-macroglobulin-like protein 1	LCMS	Liquid chromatography/mass spectrometry
CD14	Cluster of differentiation protein 14	NEC	Necrotizing enterocolitis
CST3	Cystatin 3	PEDF	Pigment epithelium-derived factor
ELISA	Enzyme-linked immunosorbent assay	RET4	Retinol binding protein 4
FGA	Fibrinogen alpha chain	ROC	Receiver-operator characteristic
IL	interleukin	VASN	Vasolin

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Methods

This was a multi-institutional, multiyear study with prospective data collection performed from May 1, 2007, to August 1, 2012, by trained personnel at each participating institution. Patient contributions by institution included: Yale-New Haven Children's Hospital (n = 42), Johns Hopkins Children's Center (n = 27), Texas Children's Hospital (n = 25), Lucile Packard Children's Hospital (n = 18), and Children's Hospital of Philadelphia (n = 7). Informed consent was obtained from the parents of all enrolled subjects. This study was approved by the human subjects' protection program at each participating institution.

All urine samples were collected at the time of initial clinical concern for disease (NEC or sepsis), a point at which definitive diagnosis was not able to be determined on clinical grounds alone. Patients with a previous diagnosis of NEC or sepsis, a history of previous abdominal surgery, or a known congenital anomaly of the gastrointestinal tract or abdominal wall were excluded from the study. Patient inclusion was ultimately confirmed by the presence of signs specific for NEC by Bell's criteria (pneumatosis intestinalis) or, for the sepsis group, by either positive blood cultures or a clinical syndrome associated with a high probability of infection. Control subjects were identified as premature infants in the neonatal intensive care unit without known or suspected inflammatory disease.

The study was conducted in 2 phases. The "discovery phase" included urine proteomics analysis by nontargeted, liquid chromatography/mass spectrometry (LCMS) with case and control subjects (n = 45 NEC, n = 12 sepsis, n = 2, controls).[16,17] To verify the LCMS spectral counts in a proof-of-principle experiment, the cluster of differentiation protein 14 (CD14) LCMS analyte results were compared with CD14 western blot analysis. For the western blot analysis, CD14 MaxPab mouse polyclonal antibody (B01; Abnova, Taipei City, Taiwan) was used as the primary antibody and a fluorescent-labeled secondary antibody was subsequently applied. Gel band intensities were quantified using GelAnalyzer software (http://www.gelanalyzer.com).

The "validation phase" consisted of the analysis of a second, naïve patient cohort (n = 40 NEC, n = 5 sepsis, n = 15 healthy controls) for which enzyme-linked immunosorbent assay (ELISA) technology was used to quantify the previously identified urine protein biomarker candidates. All ELISAs were performed according to vendor instructions for the measurement of selected biomarkers in the urine using commercially available kits (Abcam, Cambridge, Massachusetts; Biolegend Inc., San Diego, California; Ebioscience Inc., San Diego, CA; Fisher Scientific, Rockford, Illinois; and Uscn Life Science Inc., Wuhan, China). The protein analytes' urine abundance was reported as a normalized ratio of the ELISA-derived concentration to urinary creatinine concentration to correct for urine biological variations.

Statistical Analyses

Patient demographic data were analyzed using the Epidemiological calculator (R epicalc package; http://cran.r-project.org/web/packages/epicalc/index.html). Student *t* test was performed to calculate *P* values for continuous variables, and Fisher exact test was used for comparative analysis of categorical variables. Hypothesis testing to detect statistical differences in discovered biomarkers was performed using a Student *t* test (2-tailed) and Mann-Whitney *U* test (2-tailed), along with local false discovery rate[17] methods to correct for multiple hypothesis testing issues.

We then performed biomarker feature selection and panel optimization with the aim to develop a multiplexed antibody-based assay for both the diagnosis and prognosis of NEC. This was accomplished using a genetic algorithm (R genalg package; http://cran.r-project.org/web/packages/genalg/index.html) to construct biomarker panels from the validated urine protein biomarkers. Using the validation ELISA data, we identified the optimal biomarker panels by testing all possible combinations of the validated urine protein biomarkers while balancing the need for small panel size, accuracy of classification, goodness of class separation (NEC vs sepsis, medical NEC vs surgical NEC, NEC vs control, and sepsis vs control), and sufficient sensitivity and specificity.

The predictive performance of each biomarker panel analysis was evaluated by receiver-operator characteristic (ROC) curve analysis by plotting the sensitivity vs 1-specificity.[18-20] The biomarker panel score was defined as the ratio between the geometric means of the respective up- and down-regulated protein biomarkers. To define the performance of the biomarker panels we chose the coordinates on the ROC curve that represented the "cut-off" point with the best sensitivity and specificity as previously described.[19]

Results

The only patient characteristic with a statistically significance difference between groups in the discovery cohort was race, with a greater percentage of black infants in the NEC group compared with the sepsis and control groups (**Table I**). The characteristics with statistically significance differences between groups in the biomarker validation cohort were gestational age and birth weight, with infants in the control group tending to have younger gestational ages and lower birth weights than those in the NEC and sepsis groups. The time between initial clinical concern (ie, the time of urine sample collection) and confirmed medical NEC, defined as the presence of pneumatosis, was median 32 hours (IQR 9.5-66.5). The time between initial clinical concern and confirmation of surgical NEC, defined as the time of laparotomy, peritoneal drain, or death from complication of NEC, was median 48 hours (IQR 12-171.5).

Biomarker discovery (LCMS)

LCMS analysis of urine from the 59 infants in the biomarker discovery cohort revealed 13 candidate proteins