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; ■: ■-■).*

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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Funded by the Lucile Packard Foundation for Children's Health, the Food and Drug Administration (1UO1FD004194-01) [to K.S.], Ikaria Inc, the INSPIRE Center at Nationwide Children's Hospital, the Jack and Marion Euphrat Fellowship in Pediatric Translational Medicine, and Stanford University (CTSA UL1 RR025744 to Z.K.). The authors declare no conflicts of interest.

 http://dx.doi.org/10.1016/j.jpeds.2013.10.091

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

Table I. Patient characteristics

Discovery cohort (n = 59)	NEC			Sepsis (n = 12)	Control (n = 2)
	Medical NEC (n = 29)	Surgical NEC (n = 16)	Total NEC (n = 45)		
No. Obs.	n = 26	n = 14	n = 40	n = 12	n = 2
Sex					
Female	12 (46.2%)	7 (50.0%)	19 (47.5%)	7 (58.3%)	2 (100.0%)
Male	14 (53.8%)	7 (50.0%)	21 (52.5%)	5 (41.7%)	0 (0.0%)
Race*					
Asian	1 (3.4%)	0 (0.0%)	1 (5.0%)	1 (8.3%)	2 (100.0%)
Black	8 (27.6%)	5 (31.2%)	13 (28.9%)	0 (0.0%)	0 (0.0%)
White	16 (55.2%)	6 (37.5%)	22 (48.9%)	11 (91.7%)	0 (0.0%)
Unknown	4 (13.8%)	5 (31.5%)	9 (20.0%)	0 (0.0%)	0 (0.0%)
Gestational age, wk					
Median (IQR)	28.5 (27-32)	28.5 (25-31.8)	28.5 (27-32)	28 (26.5-32.5)	30.5 (28.2-32.8)
Birth weight, g					
Median (IQR)	1095 (938-1952)	970 (740.5-1771.2)	1070 (850-1947.8)	1047.5 (840-1927.5)	1840 (1350-2330)
Birth length, cm					
Median (IQR)	36 (33-42)	34.5 (33-43.2)	35.75 (33-43.2)	37 (32-43)	41 (34-48)
Birth head circumference, cm					
Median (IQR)	26 (24.5-31)	24.5 (23.5-27.9)	26 (23.5-30.2)	24.5 (24-28.8)	28.5 (26.2-30.8)

Validation cohort (n = 59)	NEC			Sepsis (n = 5)	Control (n = 15)
	Medical NEC (n = 30)	Surgical NEC (n = 10)	Total NEC (n = 40)		
Sex					
Female	16 (53.3%)	2 (20.0%)	18 (45.0%)	3 (60.0%)	6 (40.0%)
Male	14 (46.7%)	8 (80.0%)	22 (55.0%)	2 (40.0%)	9 (60.0%)
Race					
Asian	2 (6.7%)	0 (0.0%)	2 (5.0%)	0 (0.0%)	0 (0.0%)
Black	13 (43.3%)	3 (30.0%)	16 (40.0%)	3 (60.0%)	7 (46.7%)
White	13 (43.3%)	6 (60.0%)	19 (47.5%)	1 (20.0%)	7 (46.7%)
Unknown	2 (6.7%)	1 (10.0%)	3 (7.5%)	1 (20.0%)	1 (6.7%)
Gestational age, wk*					
Median (IQR)	30 (27-33)	27.5 (25-32)	29.5 (27-32.5)	28 (26-31.5)	26 (25-27.5)
Birth weight, g*					
Median (IQR)	1265 (935-1873.5)	1285 (796.5-1912.5)	1265 (907-1943.8)	950 (900-961)	730 (632.5-937.5)
Birth length, cm					
Median (IQR)	37 (34.1-41.8)	34.5 (32-42.8)	37 (32.9-42.2)	34 (31-36)	33.8 (32-36)
Birth head circumference, cm					
Median (IQR)	27.2 (25-30.5)	24.4 (23-28)	27 (23.9-30.1)	24.2 (23.4-24.6)	23 (21.8-26)

Obs, observations.
*$P < .05$.

with potentially relevant biologic roles: alpha-2-macroglobulin-like protein 1 (A2ML1), apolipoprotein CIII, complement component protein 3, caspase protein 8, CD14, cystatin 3 (CST3), fibrinogen alpha chain (FGA), kininogen protein 1, lectin manose-binding protein 2, pigment epithelium-derived factor (PEDF), Pmp-like secreted protein 2, retinol binding protein 4 (RET4), and vasolin (VASN).

As a verification of the LCMS discovery approach, the differential presence of CD14, a pattern recognition receptor, was confirmed by Western blot analysis comparing medical NEC, surgical NEC, and sepsis urine samples (**Figure 1**; available at www.jpeds.com). Western blot revealed the alpha-form and beta-form of soluble CD14, both of which are known to be up-regulated in the plasma of adults experiencing proinflammatory conditions.[21] LCMS spectral counts were then plotted against CD14 Western blot band intensity revealing a correlation coefficient of 0.86 ($P < .001$; **Figure 2**; available at www.jpeds.com) with the more severe pathology (surgical NEC) having greater levels of CD14 expression by both analytical methods.

Biomarker Validation (ELISA)

The urine samples from the 60 infants in the validation cohort were used for ELISA-based validation of the 13 candidate biomarkers. Seven of the 13 LCMS candidate biomarkers were quantitatively validated (2-tailed Mann-Whitney *U* tests, $P < .05$; **Tables II** and **III**; **Table III**, available at www.jpeds.com) and consistently shared the same trend of up- or down- regulation between case and control samples when comparing discovery LCMS and validation ELISA results. In addition, individual ROC curves were plotted for each validated analyte and the point of intersection for optimal sensitivity and specificity was computed, demarcated, and reported (**Figure 3** and **Table IV**; available at www.jpeds.com).

The genetic algorithm panel construction process led to the design of four distinct biomarker panels with complete separation between NEC vs sepsis, medical NEC vs surgical NEC, NEC vs control, and sepsis vs control (**Table V** and **Figure 4**). These biomarker panels are nonredundant, indicative of their noninclusive relationships.

Table II. ELISA biomarker validation by Mann-Whitney *U* test

	Mann-Whitney *U* test *P* value			
Analyte	NEC M vs NEC S	NEC vs Sepsis	NEC vs control	Sepsis vs control
A2ML1	.02*	.08	1.40×10^{-4}†	.50
CD14	.02*	.77	.12	.35
CST3	.12	.58	.03*	.35
FGA	.02*	.8	.06	.16
PEDF	1.82×10^{-3}†	.03*	2.23×10^{-4}†	.67
RET4	6.89×10^{-3}	.64	.11	.50
VASN	.09	.80	.02*	.12

NEC M, medical NEC; *NEC S*, surgical NEC.

Importantly, each biomarker panel was able to differentiate between the groups with sensitivities ranging from 0.89 to 0.96 and specificity ranging from 0.80 to 0.90 (**Figure 4**). Not surprisingly, the panels assessing infants with diagnoses more closely related in severity of inflammation had lower sensitivity (NEC vs sepsis, 0.89; and medical NEC vs surgical NEC, 0.89) compared with the panels including the controls (NEC vs control, 0.96; and sepsis vs control, 0.90).

Discussion

Considerable effort has been directed toward the identification of biomarkers of NEC given the inability to predict the ultimate course of disease based on clinical variables alone.[22] Exploratory proteomics enables the unbiased identification of candidate biomarkers before clinical manifestation of disease. Urine biomarker panels, specifically, hold the potential to provide low-risk, low-cost facilitation of clinical decision-making. The urine protein biomarkers described in the current study enabled the accurate diagnosis of NEC amongst a population of infants with NEC, infants with non-NEC sepsis, and noninfected premature infants. In addition, these biomarkers showed potential prognostic value, as they were also able to accurately differentiate between infants with medical NEC and those with surgical NEC.

Many previous studies have investigated the diagnostic capabilities of targeted biomarkers for NEC. Epidermal growth factor,[23,24] interalpha inhibitor proteins,[25-28] intestinal fatty acid-binding protein,[29-33] and fecal calprotectin,[34] have all been identified as potential biomarkers of NEC in human infants. In addition, a number of interleukins (ILs) and other inflammatory factors are either up-regulated (IL 1, 6, 8, and 12, tumor necrosis factor-alpha, interferon, and platelet-activating factor), down-regulated, or temporally correlated with the severity of disease (IL 4, 10, and 11) in infants with NEC or other inflammatory conditions of infancy.[35-42] Despite promising results, no single biomarker has proven to be useful as a stand-alone diagnostic test in clinical practice. In contrast, the current study made use of a nontargeted, exploratory approach to identify several candidate biomarkers. The biomarker panels were subsequently validated on a naïve population with relatively strong diagnostic (NEC vs sepsis; mean area under the curve 98.2%, sensitivity 0.89, specificity 0.80) and prognostic (medical NEC vs surgical NEC; mean area under the curve 98.4%, sensitivity 0.89, specificity 0.90) capabilities.

Importantly, many of these have potential physiologic bases for their association with NEC. Alpha-2macroglobulin (which shares significant homology with A2ML1) and FGA are both components of the coagulation cascade, a potentially significant finding given that coagulation necrosis is a common pathologic finding in NEC resection specimens. VASN is an inhibitor of transforming growth factor-beta, and is down-regulated after vascular injury,[43] a finding consistent with lower urine levels of VASN in the surgical NEC cohort. The pattern recognition receptor CD14 is a regulator of the innate immune system that plays a role in the response to bacterial lipopolysaccharide, potentially explaining its elevation in the surgical NEC cohort, a patient group with more extensive bowel injury and thus bacterial invasion. CST3 has been described as a biomarker for acute kidney injury,[44] likely explaining its presence in greater levels in the urine as systemic disease progresses. Although these associations are intriguing, further investigation is needed to identify causal relationships and to provide further biologic insight.

This study demonstrates the utility of unbiased biomarker discovery platforms in which proteins with correlated and potentially causal relationships to the pathophysiology of disease can be identified. The clinical potential of the described biomarker panel was highlighted by the validation on a naïve population, even though the inclusion of the sepsis group in addition to the noninfected control group confirmed that the identified biomarkers were not simply markers of a generic proinflammatory state.

A significant limitation of our approach, indeed a characteristic inherent to any proteomics-based study, was the absence of a clear pathophysiologic link between the identified biomarkers and the pathology in question. As previously

Table V. Biomarker panels for NEC M vs NEC S, NEC vs control, NEC vs sepsis, and sepsis vs control classifications

	Analyte							Sample panel score			Sample panel score	
Clinical usefulness	A2ML1	CD14	CST3	FGA	PEDF	RET4	VASN	Class	Median (IQR)	Mean (SD)	Class	Median (IQR)	Mean (SD)
NEC M vs NEC S	+↑	+↓	+↓	-	+↓	+↓	+↑	NEC M	2.1 (0.8-2.5)	2.64 (3.60)	NEC S	125.5 (28.9-208.9)	118.9 (105.7)
NEC vs Control	-	-	+↑	-	+↓	+↑	-	NEC	19.4 (9.7-67.9)	161.35 (596.71)	Control	0.4 (0.23-1.1)	0.8 (0.8)
NEC vs Sepsis	-	-	+↑	-	+↓	+↑	-	NEC	19.4 (9.7-67.9)	161.35 (596.71)	Sepsis	1.4 (0.49-1.9)	1.3 (1.1)
Sepsis vs control	+↑	-	+↑	+↑	-	-	+↑	Sepsis	21.2 (20.4-48.1)	38.60 (31.50)	Control	4.3 (2.26-12.8)	10.4 (13.0)

Sample panel score was defined as the ratio of the geometric mean of the up-regulated panel markers' assay results and those of the down-regulated panel markers' assay results.

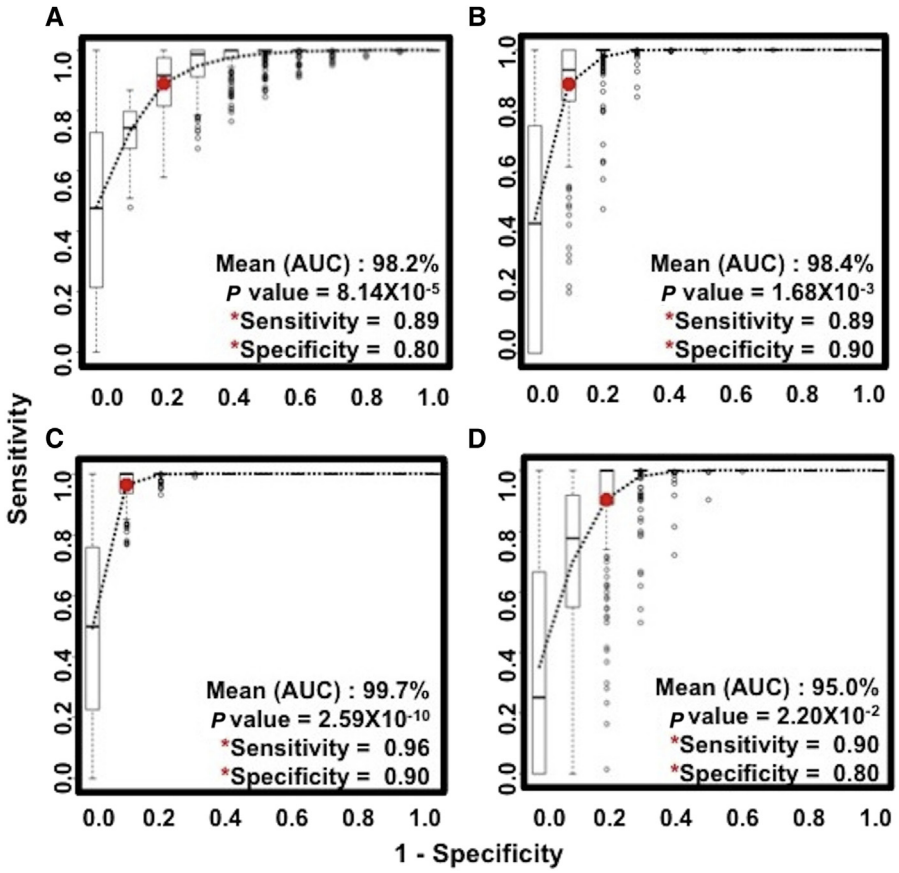

Figure 4. Biomarker panel ROC curves. The *black dots* represent "cut-off" points along the ROC curves, indicating the best sensitivity and specificity coordinates. **A,** NEC vs sepsis consists of 3 proteins: CST3, PEDF, and RET4. **B,** Medical NEC vs surgical NEC consists of 6 proteins: A2ML1, CD14, CST3, PEDF, RET4, and VASN. **C,** NEC vs control consists of 3 proteins: CST3, PEDF, and RET4. **D,** Sepsis vs control consists of 4 proteins: A2ML1, CST3, FGA, and VASN. *Significant and highly significant. *AUC*, area under the curve.

mentioned, many of the biomarkers identified and validated in this study are related to known coagulation, inflammatory, or immunologic cascades. Although not confirming causal significance, this is an interesting finding that requires additional study.

Another important limitation of this pilot study is the relatively small cohort size illustrating the need for further prospective validation and longitudinal testing. Furthermore, there were noted differences in, and thus possible confounding of our results by, gestational age and birth weight between the study groups in the biomarker validation cohort. Future studies will be needed to address impact of these factors on biomarker validity. Despite such limitations, the quantitative differences in urine biomarker levels between multiple study groups validated on a naïve population suggest potential future clinical utility.

The use of an unbiased exploratory proteomics approach to identify urine biomarkers for NEC led to the development of a panel of validated proteins that demonstrate promise as a clinically useful instrument. The incorporation of additional targeted biomarkers along with patient-specific clinical information will likely strengthen the utility of the described biomarkers and is an important area of ongoing investigation. With continued refinement, it appears likely that a biomarker-based instrument will lead to more efficient diagnosis, more timely intervention, and improved outcomes for infants affected by one of the most common and debilitating diseases of prematurity. ■

Submitted for publication Apr 20, 2013; last revision received Aug 21, 2013; accepted Oct 14, 2013.

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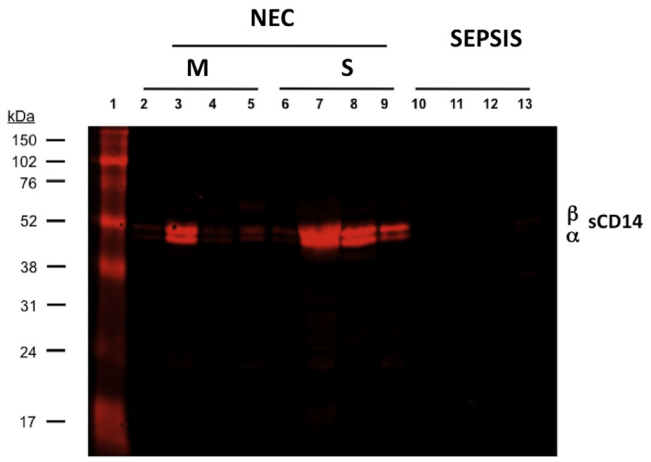

Figure 1. Western blot analysis of urine CD14. *M*, medical, *S*, surgical; *sCD-14*, soluble CD14.

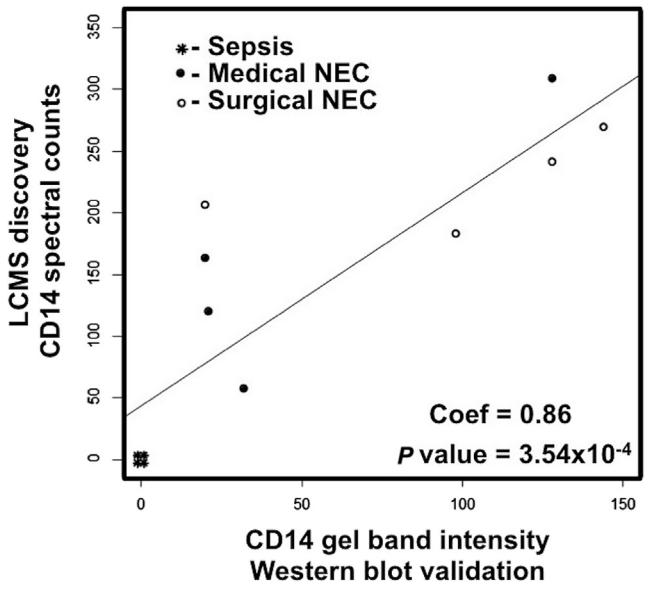

Figure 2. Correlation of CD14 LCMS spectral counts and CD14 Western blot gel band intensity for infants in the sepsis, medical NEC, and surgical NEC groups. *Coef*, coefficient.

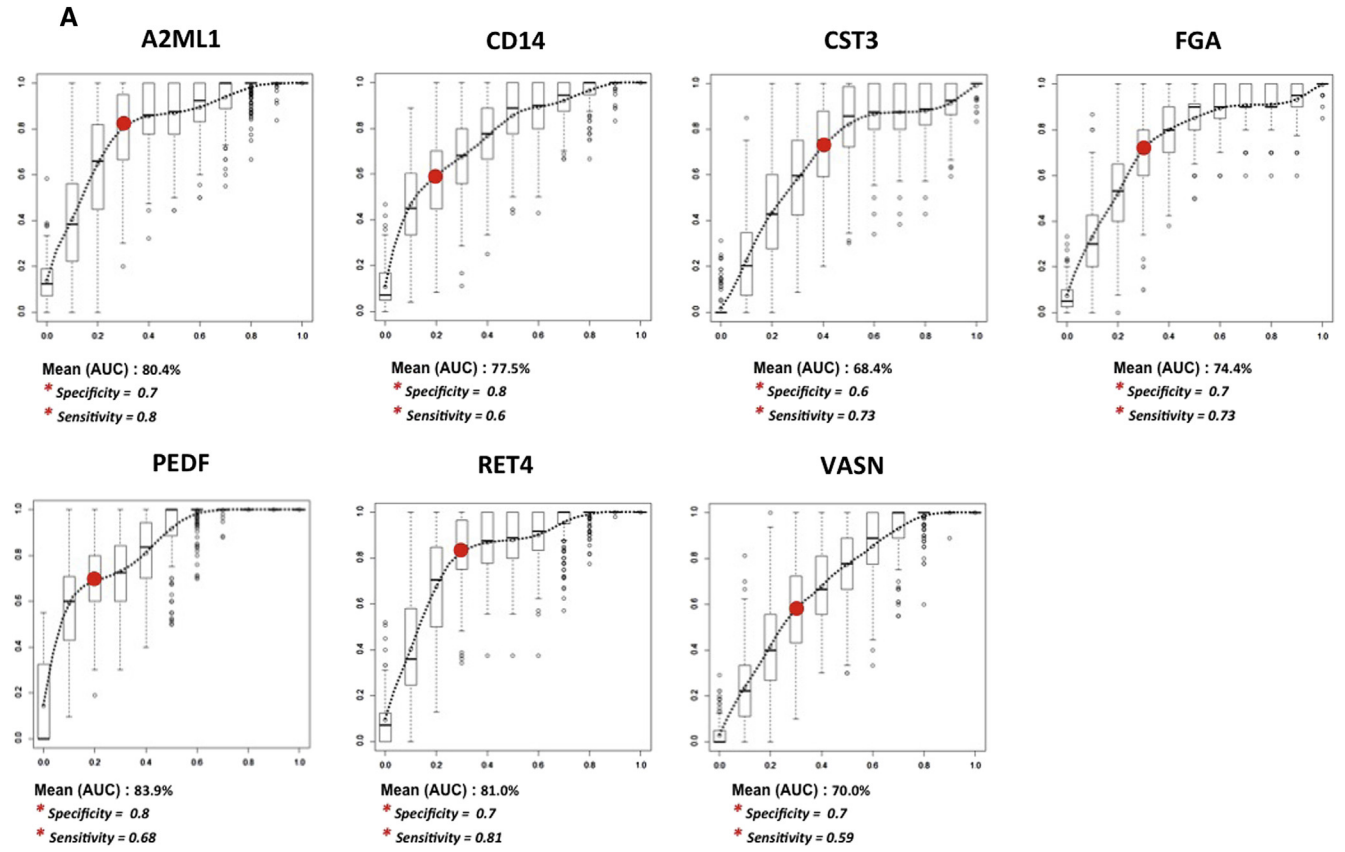

Figure 3. Single analyte biomarker's performances in discriminating **A,** medical and surgical NEC; **B,** NEC and control; **C,** NEC and sepsis; and **D,** sepsis and control classes were analyzed by ROC analysis. The Y-axis is the sensitivity and X-axis is the 1 − specificity. The *red dot* represents the point of optimized sensitivity and specificity and is listed under each ROC plot. A total of 500 testing data sets were generated by bootstrapping methods from the ELISA data and were used to derive estimates of SE and CIs for the ROC analyses. The plotted ROC curve represents the vertical average of the 500 bootstrapping runs, and the box and whisker plots show the vertical spread around the average. *(Continues)*

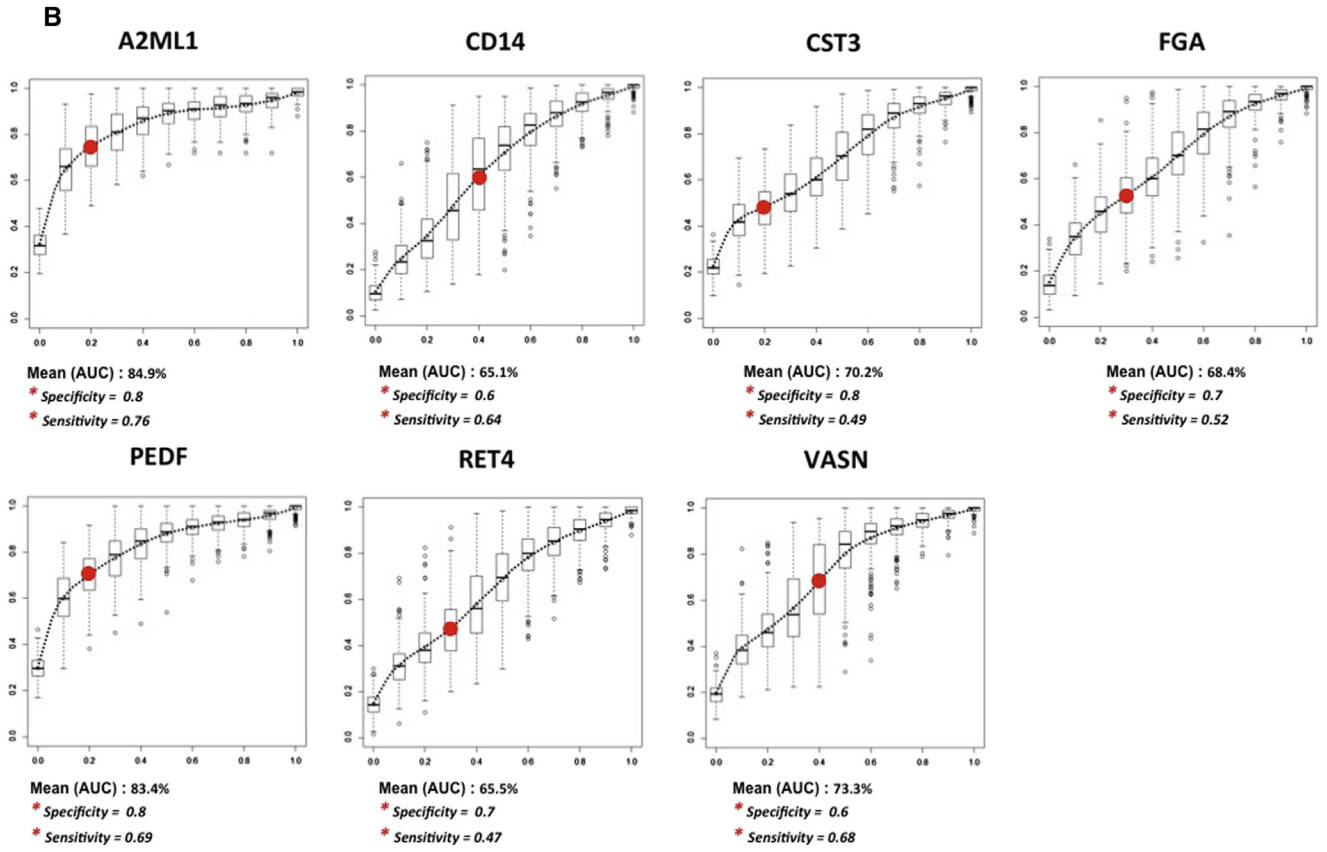

Figure 3. Continues.

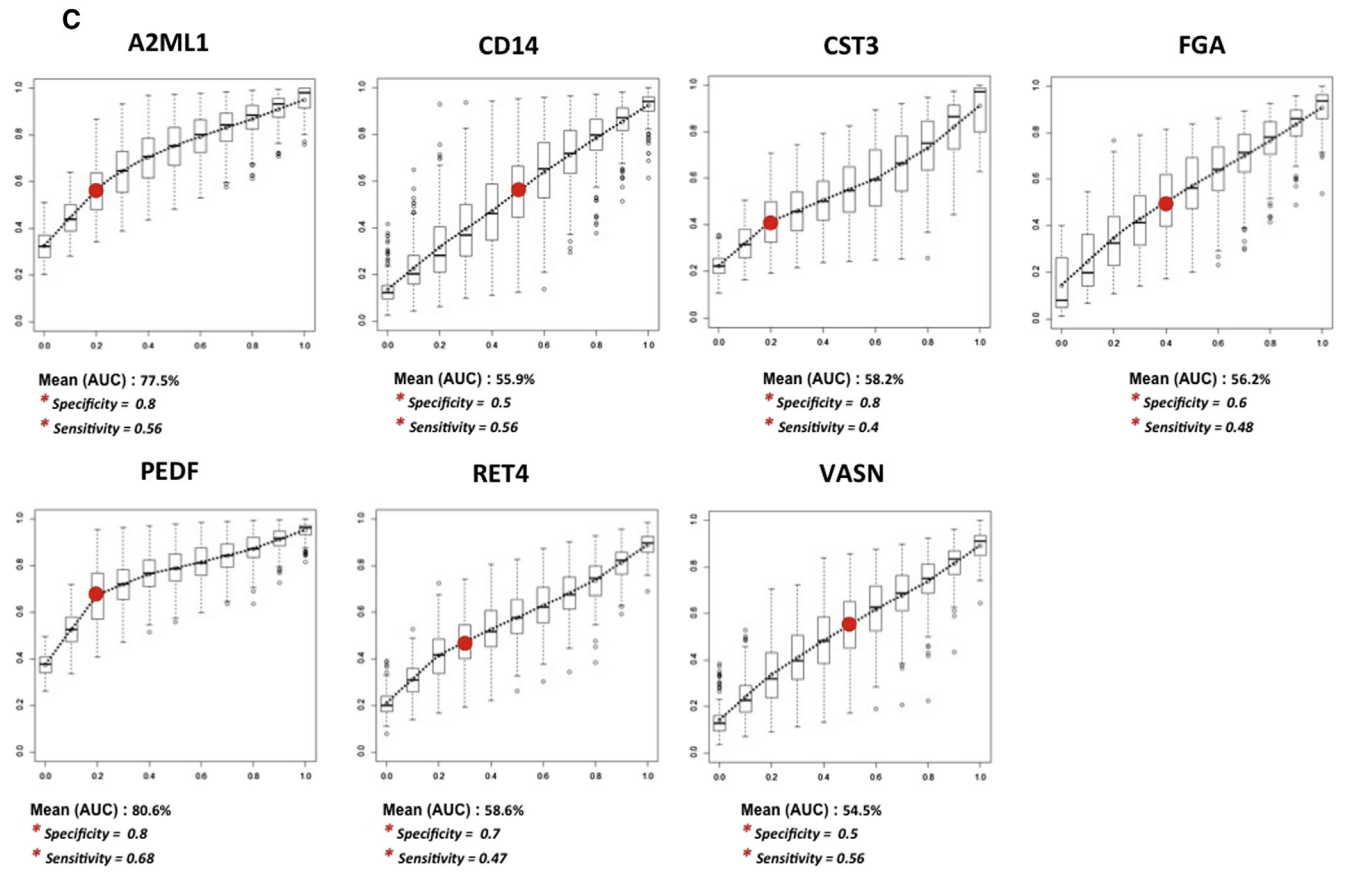

Figure 3. Continues.

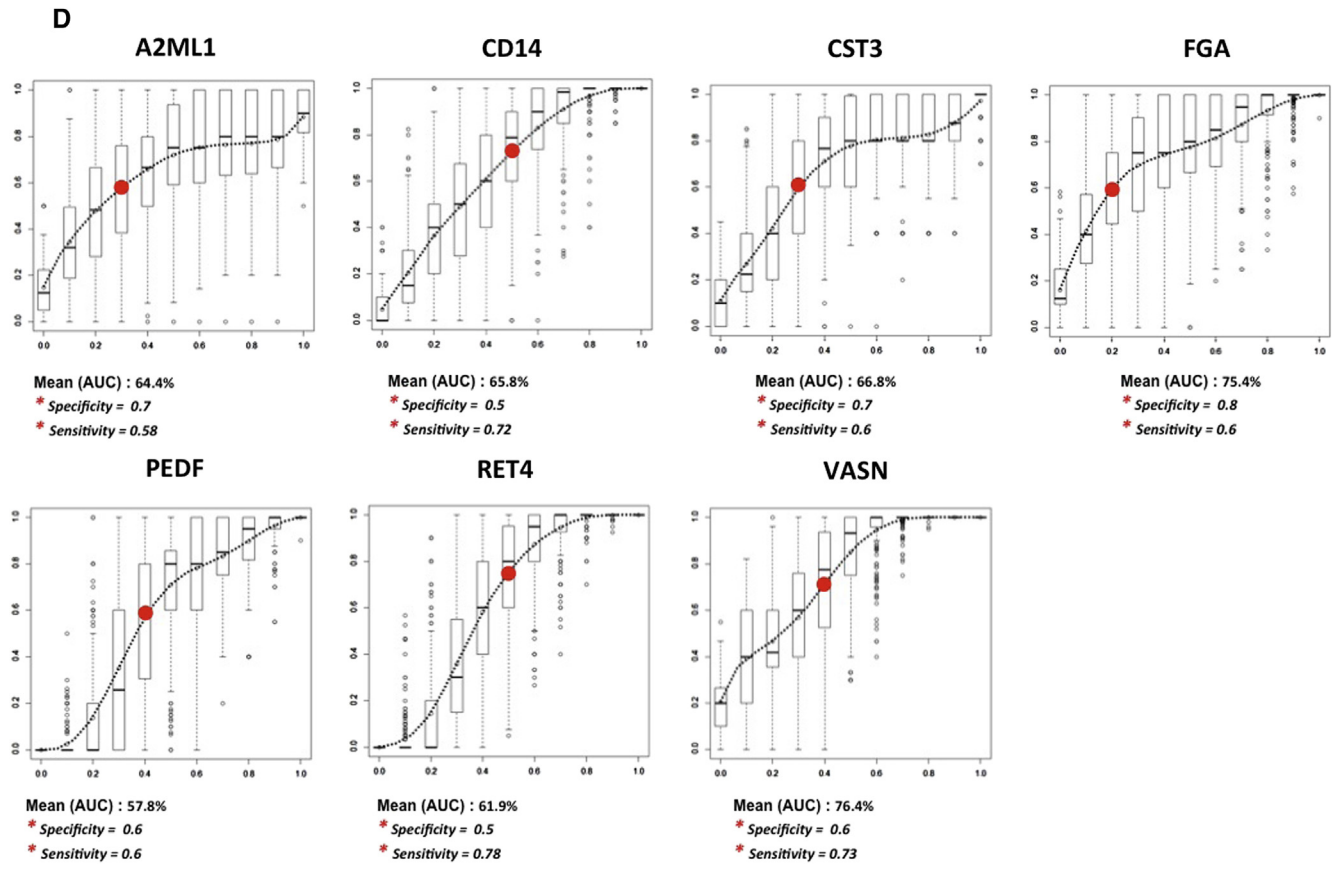

Figure 3. Continued.

Table III. Validated biomarker levels by pathologic group

		NEC									
		M		S		M + S		Sepsis		Control	
Analyte	Unit	Median (IRQ)	Mean (SD)	Median (IRQ)	Mean (SD)	Median (IRQ)	Mean (SD)	Median (IRQ)	Mean (SD)	Median (IRQ)	Mean (SD)
A2ML1	Analyte/Cr, ng/mg	61.55 (14.12-166.37)	174.03 (346.64)	3.79 (1.40-9.51)	22.25 (47.81)	28.28 (3.79-130.13)	138.61 (309.67)	3.32 (1.55-9.06)	5.30 (6.37)	1.68 (0.96-3.36)	2.71 (3.03)
CD14	Analyte/Cr, ng/mg	174.40 (84.74-524.22)	451.76 (726.18)	895.49 (231.43-2601.20)	2740.24 (5004.98)	212.66 (110.28-679.12)	979.87 (2574.92)	186.53 (100.67-655.39)	367.62 (361.03)	89.44 (39.14-574.68)	295.63 (375.31)
CST4	Analyte/Cr, ng/mg	43.70 (21.30-225.23)	215.50 (416.28)	227.20 (120.54-605.62)	355.27 (352.39)	87.30 (23.16-239.16)	248.39 (401.55)	94.22 (59.22-111.59)	81.14 (52.93)	31.14 (12.89-86.68)	51.12 (47.37)
FGA	Analyte/Cr, ng/mg	15.78 (9.26-33.81)	74.18 (143.97)	69.50 (46.25-237.97)	408.39 (862.69)	21.57 (9.95-97.57)	157.73 (456.77)	29.06 (15.51-175.91)	95.71 (149.33)	15.52 (4.23-23.63)	22.67 (35.07)
PEDF	Analyte/Cr, ng/mg	4.40 (1.57-25.31)	66.05 (228.22)	122.04 (7.14-257.70)	225.45 (309.84)	8.60 (2.79-105.75)	115.86 (262.27)	111.66 (100.56-134.47)	212.40 (225.50)	217.34 (57.49-491.52)	378.60 (411.31)
RET4	Analyte/Cr, ng/mg	417.89 (188.59-655.45)	642.35 (846.82)	1122 (898.48-2083.34)	5549.31 (12 299.56)	512.72 (197.95-1115.57)	1796.93 (6090.69)	454.38 (337.35-655.21)	463.24 (220.19)	298.60 (115.29-692.03)	406.36 (357.47)
VASN	Analyte/Cr, ng/mg	23.93 (9.78-129.94)	97.17 (163.15)	9.8 (6.32-21.43)	17.04 (18.11)	19.99 (9.04-52.85)	78.68 (146.81)	13.67 (10.70-43.44)	26.40 (24.62)	2.74 (0.54-22.83)	11.04 (12.62)

Cr, creatinine.

Table IV. Individual biomarker intercohort testing characteristics

	NEC M vs NEC S			NEC vs control			NEC vs sepsis			Sepsis vs control		
Analyte	ROC AUC	Sensitivity*	Specificity*	ROC AUC	Sensitivity*	Specificity*	ROC AUC	Sensitivity*	Specificity*	ROC AUC	Sensitivity*	Specificity*
A2ML1	80.40%	0.80	0.70	84.90%	0.76	0.80	77.50%	0.56	0.80	0.78	0.56	0.80
CD14	77.50%	0.60	0.80	65.10%	0.64	0.60	55.90%	0.56	0.50	0.56	0.56	0.50
CST4	68.40%	0.73	0.60	70.20%	0.49	0.80	58.20%	0.4	0.80	0.58	0.40	0.80
FGA	74.40%	0.73	0.70	68.40%	0.52	0.70	56.20%	0.48	0.60	0.56	0.48	0.60
PEDF	83.90%	0.68	0.80	83.40%	0.69	0.80	80.60%	0.68	0.80	0.58	0.60	0.60
RET4	81.00%	0.81	0.70	65.50%	0.47	0.70	58.60%	0.47	0.70	0.62	0.78	0.50
VASN	70.00%	0.59	0.70	73.30%	0.68	0.60	54.50%	0.56	0.50	0.76	0.73	0.60

AUC, area under the curve.
*The optimal sensitivity and specificity point along the ROC curve.