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ORIGINAL ARTICLE

A novel urine peptide biomarker-based algorithm for the prognosis of necrotising enterocolitis in human infants

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ABSTRACT

Objective Necrotising enterocolitis (NEC) is a major source of neonatal morbidity and mortality. The management of infants with NEC is currently complicated by our inability to accurately identify those at risk for progression of disease prior to the development of irreversible intestinal necrosis. We hypothesised that integrated analysis of clinical parameters in combination with urine peptide biomarkers would lead to improved prognostic accuracy in the NEC population.

Design Infants under suspicion of having NEC (n=550) were prospectively enrolled from a consortium consisting of eight university-based paediatric teaching hospitals. Twenty-seven clinical parameters were used to construct a multivariate predictor of NEC progression. Liquid chromatography/mass spectrometry was used to profile the urine peptidomes from a subset of this population (n=65) to discover novel biomarkers of NEC progression. An ensemble model for the prediction of disease progression was then created using clinical and biomarker data.

Results The use of clinical parameters alone resulted in a receiver-operator characteristic curve with an area under the curve of 0.817 and left 40.1% of all patients in an 'indeterminate' risk group. Three validated urine peptide biomarkers (fibrinogen peptides: FGA1826, FGA1883 and FGA2659) produced a receiver-operator characteristic area under the curve of 0.856. The integration of clinical parameters with urine biomarkers in an ensemble model resulted in the correct prediction of NEC outcomes in all cases tested.

Conclusions Ensemble modelling combining clinical parameters with biomarker analysis dramatically improves our ability to identify the population at risk for developing progressive NEC.

Significance of this study

What is already known about this subject?

- Necrotising enterocolitis is a leading cause of morbidity and mortality in premature infants.
- Early identification of infants at high risk for progressive disease is needed to improve patient outcomes and the efficiency of resource use.
- Clinical parameters alone are too inaccurate to reliably risk stratify affected infants.
- Multiple attempts to define molecular indicators of disease progression by targeted approaches have been unsuccessful.

What are the new findings?

- A prognostic algorithm based on clinical parameters alone placed 40.1% of all infants in an 'indeterminate' risk group.
- An unbiased exploratory proteomics platform led to the identification of three urine fibrinogen peptides (FGA1826, FGA1883 and FGA2659) predictive of progressive disease.
- The integration of clinical parameters with these urine biomarkers in an ensemble model resulted in the correct prediction of progressive disease in all cases tested.

How might it impact on clinical practice in the foreseeable future?

- The ensemble model holds the potential to focus the concentration of clinical care on high-risk infants. This instrument will also potentially guide the implementation of novel preventative or curative therapies as they are developed.

INTRODUCTION

Necrotising enterocolitis (NEC) is one of the leading causes of morbidity and mortality in premature infants. It occurs in 1–3/1000 births and in up to 15% of very low birthweight infants.[1 2] About a half of infants with NEC will recover with medical therapy (medical NEC) while the remainder will develop intestinal perforation and/or necrosis requiring emergency surgical intervention (surgical NEC).[3 4] Approximately a half of those who develop progressive disease will die and the remainder will have a high rate of long-term disability.

NEC occurs in an unpredictable manner, without identifiable triggering events.[5] The lack of ability to predict which infants with NEC will develop progressive disease makes it difficult to identify a target population for preventative therapies or novel treatment strategies. Initial attempts to create

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prognostic algorithms based on clinical data alone have not been successful leading to interest in the development of biomarker-based prognostic instruments.[6]

High-throughput molecular analysis has identified biomarkers that have proven to be predictive of outcomes in many human diseases. To date, however, investigators have not identified individual biological indicators of NEC that have sufficient predictive value to be clinically adopted. We hypothesised that the integrated analysis of patient-specific clinical parameters and urine peptide biomarkers would improve our ability to identify high-risk infants prior to the development of irreversible disease progression.

MATERIALS AND METHODS

Patient population

This study was approved by the human subjects' protection programme at each participating institution (*Stanford protocol ID 23091*). Informed consent was obtained from the parents of all enrolled subjects. Patient contributions by institution included: Baylor/Texas Children's Hospital (n=184), Yale-New Haven Children's Hospital (n=158), UCSF/Benioff Children's Hospital (n=79), Boston Children's Hospital (n=75), UCLA/Mattel Children's Hospital (n=42), Johns Hopkins Children's Center (n=22), Stanford/Lucile Packard Children's Hospital (n=16) and Children's Hospital of Philadelphia (n=11). Complete data collection including patient-specific demographic, clinical and laboratory data were prospectively collected from a total of 550 infants. Those with incomplete data collection were excluded from the study.

Urine samples were collected from a subset of 65 infants with suspected NEC. Patient contributions by institution included: Baylor/Texas Children's Hospital (n=16), Yale-New Haven Children's Hospital (n=24), Johns Hopkins Children's Center (n=17), Stanford/Lucile Packard Children's Hospital (n=4) and Children's Hospital of Philadelphia (n=4). The samples were collected at the time of initial clinical concern for NEC. The infants were then followed clinically and ultimately categorised as either medical NEC (improved without surgery) or surgical NEC (required laparotomy, peritoneal drainage or died from complications of NEC prior to intervention). The urine samples were then compared between the medical NEC and surgical NEC groups for all subsequent analyses.

For the development of the clinical parameter-based prognostic algorithm 485 infants were randomised into two cohorts for statistical training (n=323) and testing (n=162). For the urine peptidome analysis the remaining 65 infants were assigned to either the biomarker discovery cohort (n=28) or the biomarker validation cohort (n=37). Comparative demographic analyses were performed by Cochran Mantel-Haenszel χ^2 and analysis of variation (ANOVA) with adjustment for institution (R epicalc package).

Clinical parameter-based prognostic algorithm

The clinical parameters for the infants randomised to the statistical training cohort (n=323) were analysed by linear discriminant analysis (LDA) using the R library MASS function 'lda' (http://www.r-project.org/). All subjects in the training cohort were subsequently assigned to one of three possible subgroups (low-risk, indeterminate or high-risk) based on 95% correct classification in the low-risk (5% probability of actually being surgical NEC) and in the high-risk (5% probability of actually being medical NEC) groups. This process was then repeated on the testing cohort (n=162). The prognostic characteristics of the clinical parameters were then subjected to receiver-operator characteristic (ROC) analyses of their ability to differentiate infants with medical NEC from those with surgical NEC.

Urine biomarker-based prognostic algorithm

Biomarker discovery and validation overview

We assigned 65 subjects to either the biomarker discovery cohort (n=28) or the biomarker validation cohort (n=37). Urine sample collection, processing and peptide extraction were performed according to previously described protocols.[7–9] We used liquid chromatography—matrix-assisted laser desorption/ionisation mass spectrometry (MS) (LC-MALDI, ABI 4700, Applied Biosystems, California, USA) for comprehensive analysis of the urine peptidomes. Biomarker validation was performed by repeat, confirmatory analysis of the initial 28 infants in the discovery cohort followed by analysis of the 37 patients in the naïve validation cohort using multiple reaction monitoring (MRM) assays conducted on a triple quadrupole mass spectrometer (Quattro Premier, Waters Corporation, Massachusetts, USA).

Details of biomarker discovery

We performed a comprehensive urine peptidome analysis using a label-free approach. This entailed first selecting biomarker candidate MS peaks on the basis of discriminant analysis and then targeting candidate biomarkers for tandem MS (MS/MS) sequencing analysis to identify the peptide sequences of interest. We used our in-house informatics platform, 'MASS-Conductor' (Ling, unpublished), which consists of an integrated suite of algorithms and statistical methods to allow comprehensive analysis of LC-MALDI-based urine peptide profiling as previously described.[10–12]

To confirm the identity of the candidate peptide biomarkers we then used extensive MALDI-time of flight (TOF)/TOF and linear trap quadrapole Orbitrap MS/MS analyses coupled with database searches as previously described.[7–9 13] All features were ranked by a nearest shrunken centroid algorithm to optimise the differentiation between the medical NEC and the surgical NEC groups.[14]

Details of biomarker validation

Following the discovery of candidate peptide biomarkers, we developed and performed MRM assays as previously described.[9] Stable isotope-labelled peptides (with a ^{13}C-labelled amino acid) were synthesised and used as internal standard peptides. MRM measurement was normalised to each sample's total peptide content (TNBS assay) for further data analysis. The performance of the urine peptide classifiers using the MRM measurements were assessed and visualised by receiver-operating characteristic curve ROCR package.[15]

Ensemble algorithm combining clinical parameters and urine biomarkers

We used LDA to classify individual subjects based on clinical findings and validated urine peptides using the R library MASS function 'lda'. We performed ROC analyses of the predictive performance. The projection value onto the first canonical (LDA) was designated as the NEC outcome score, allowing the clinical parameters and fibrinogen (FGA) urine peptides to be collectively interpreted on a scale, rather than a strict binary discrimination.

RESULTS

Patient characteristics

Basic patient characteristics and demographics are shown in tables 1 and 2. Observed trends for gender, gestational age and

Table 1 Patient characteristics for necrotising enterocolitis (NEC) clinical outcome algorithm development

	Training			Testing		
	Medical NEC n=230 (71.2%)	Surgical NEC n=93 (28.8%)	p Value	Medical NEC n=115 (71.0%)	Surgical NEC n=47 (29.0%)	p Value
Male*	125 (54.4%)	61 (65.6%)	0.048	56 (48.7%)	33 (70.2%)	0.020
Gestational age (weeks)†	29.8 (29.3 to 30.3)	28.6 (27.8 to 29.4)	0.055	30.2 (29.4 to 30.9)	29.5 (28.2 to 30.7)	0.559
Birth weight (grams)†	1376.3 (1283.0 to 1469.5)	1142.1 (995.4 to 1288.8)	0.029	1418.8 (1275.4 to 1562.1)	1329.6 (1104.9 to 1554.3)	0.625
Race*			0.301			0.959
Caucasian	119 (51.7%)	41 (44.1%)		52 (45.2%)	22 (46.8%)	
African American	65 (28.3%)	31 (33.3%)		39 (33.9%)	18 (38.3%)	
Hispanic	55 (23.9%)	26 (28.0%)		27 (23.5%)	14 (29.8%)	
Asian	8 (3.5%)	2 (2.2%)		4 (3.5%)	1 (2.1%)	
Native Hawaiian or Pacific Islander	0 (0%)	2 (2.2%)		1 (0.9%)	0 (0%)	
American Indian or Alaskan Native	2 (0.9%)	0 (0%)		0 (%)	0 (0%)	
Unknown	30 (13.0%)	13 (14.0%)		16 (13.9%)	5 (10.6%)	
Other	6 (2.6%)	4 (4.3%)		3 (2.6%)	1 (2.1%)	

Patients had the opportunity to report as Hispanic in addition to the other race identifiers.
*Cochran Mantel-Haenszel χ^2 test with adjustment for different institutions is used; percentages in parentheses.
†ANOVA; least square mean is reported with 95% CI in parentheses.

birth weight exist with the surgical NEC cohorts tending to be men of younger gestational age and lower birth weight. These trends reached statistical significance only with regards to patient gender (in the clinical algorithm and biomarker groups, tables 1 and 2) and birth weight (in the clinical algorithm group, table 1), and are likely of little clinical significance as such trends are frequently observed in infants with NEC.

The time between initial clinical concern (the time of urine sample collection) and confirmed medical NEC, defined as the presence of pneumatosis was median 31 h (IQR 10, 63). 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 57 h (IQR 17, 213). There were no NEC-related deaths in the medical NEC cohort (n=389) and the combined mortality rate for the surgical NEC cohort was 27.9% (45/161).

Effectiveness of clinical parameter-based prognostic algorithm

The LDA-based model risk stratified all subjects in training and testing cohorts into the three levels of risk for progression as discussed above (low-risk, indeterminate and high-risk). Twenty-seven clinical parameters were used in the LDA analysis based on the coefficients of linear discriminants as listed in table 3. The LDA clinical risk stratification algorithm could not confidently predict the outcome for 42.4% and 40.1% of training and testing subjects respectively—percentages representing the proportion of infants remaining in the indeterminate group (figure 1A). ROC analysis and calculated area under the curves (AUCs) for the outcome prediction of medical NEC or surgical NEC were 0.894 and 0.817 in the training and testing cohorts, respectively (figure 1B).

Table 2 Patient characteristics for necrotising enterocolitis (NEC) biomarker algorithm development

	Discovery			Validation		
	Medical NEC n=17 (60.7%)	Surgical NEC n=11 (39.3%)	p Value	Medical NEC n=27 (73.0%)	Surgical NEC n=10 (27.0%)	p Value
Male*	7 (41.2%)	10 (90.9%)	0.025	12 (44.4%)	5 (50.0%)	0.763
Gestational age (weeks)†	28.9 (27.3 to 30.6)	28.0 (25.9 to 30.1)	0.236	31.9 (24.0 to 40.0)	29.4 (23.0 to 38.0)	0.873
Birth weight (grams)†	1230.5 (917.3 to 1543.7)	1167.9 (778.6 to 1557.3)	0.609	1834.9 (598.0 to 4150.0)	1470.1 (540.0 to 2951.0)	0.457
Race*			0.145			0.598
Caucasian	12 (70.6%)	4 (36.3%)		15 (55.5%)	4 (40.0%)	
African American	3 (17.7%)	5 (45.5%)		9 (33.3%)	4 (40.0%)	
Hispanic	2 (11.8%)	3 (27.3%)		0 (0%)	0 (0%)	
Asian	2 (11.7%)	0 (0%)		1 (3.7%)	0 (0%)	
Native Hawaiian or Pacific Islander	0 (0%)	0 (0%)		0 (%)	0 (0%)	
American Indian or Alaskan native	0 (0%)	0 (0%)		0 (%)	0 (0%)	
Unknown	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
Other	0 (0%)	0 (0%)		2 (7.5%)	2 (20.0%)	

Patients had the opportunity to report as Hispanic in addition to the other race identifiers.
*Fischer's exact test; percentages in parentheses.
†ANOVA; least square mean is reported with 95% CI in parentheses.

Table 3 Clinical parameters ordered by contribution (weight, LD1) to the necrotising enterocolitis (NEC) outcome linear discriminant analysis (LDA) model

Diagnostic criteria	LD1
pH value	−2.94E+00
Portal venous gas?	1.66E+00
Air/fluid levels?	7.71E−01
Thrombocytopenia	7.33E01
On a ventilator on the day protocol definition of NEC was met?	6.94E−01
Abdominal distention?	5.91E−01
Abdominal tenderness?	4.82E−01
Neutropenia	4.60E−01
Abdominal wall discoloration?	4.17E−01
Feeding intolerance?	3.95E−01
Pneumatosis intestinalis?	3.87E−01
Apneic/bradycardic episode?	−3.06E−01
Acidosis	2.69E−01
Dilated bowel?	2.59E−01
pH site	−2.34E−01
On vasopressors on the day protocol definition of NEC was met?	−1.32E−01
Capillary refill time greater than 2 s?	9.12E−02
Ileus present?	−6.67E−02
Oxygen desaturation episode?	−6.48E−02
ANC (neutrophil counts)	5.53E−02
Grossly bloody stools?	5.50E−02
WBC ($\times 10^3/mm^3$)	−2.83E−02
Thickened bowel walls?	−1.80E−02
BAND%	1.47E−02
Neutrophils (%)	1.09E−02
Bicarbonate (meg/L)	6.55E−04
Platelets (×103/μL)	3.52E−04

ANC, absolute neutrophil count; BAND, band neutrophil; LD1, coefficient of linear discriminant; WBC, white blood cell count.

Effectiveness of biomarker-based prognostic algorithm

Biomarker discovery

The MALDI-TOF MS analysis of the urine samples from the 28 infants in the biomarker discovery cohort resolved a total of 17 173 peptide peaks defined by distinct mass to charge ratio and high performance liquid chromatography fractions in the 900–4000-Da range. The nearest shrunken centroid algorithm then identified the most significant 473 peptides (sequence identified through MSMS analysis—data not shown). A LDA model was then implemented to identify a biomarker panel of optimal feature number by balancing the need for small panel size, accuracy of classification, goodness of class separation (medical NEC vs surgical NEC), and with sufficient sensitivity and specificity. This analysis revealed an optimum 36-peptide panel for which the probability scores indicated goodness of class separation for medical NEC and surgical NEC (figure 2A).

Unsupervised hierarchical cluster analysis with heat map plotting was then used to visually depict the association of the disease status with the abundance pattern of these peptides (figure 2B). This analysis demonstrated two major clusters reflecting NEC disease progression status, reinforcing the effectiveness of this urine peptide 'signature' in predicting medical NEC and surgical NEC class distinction. Student t test and Mann-Whitney U test, in addition to MSMS sequence identification (table 4) analyses were performed for these urine peptides.

We then examined the list of candidate urine peptide biomarkers and the associated signalling pathways that define their biology. Pathway analysis (figure 2C) using the PANTHER database[16] revealed the candidate peptide biomarkers to be principally involved in integrin signalling (65.7%), plasminogen activating cascade (11.4%), blood coagulation (11.4%), ubiquitin proteasome pathway (8.6%) and inflammation mediated by chemokine and cytokine signalling pathway (2.9%). Moreover, sequence alignment of the candidate peptides revealed tight sequence clusters for two fibrinogen A (FGA2560, FGA2659) and two uromodulin (UMOD 1680, UMOD 1912) peptides. We previously described two additional FGA peptides with the same N termini as FGA2560 and FGA2659 but with shorter sequences (FGA1826 and FGA1883) as candidate urine peptide biomarkers.[13] Given the biological plausibility and peptide homogeneity, these peptides were selected for further validation on the naïve biomarker validation cohort.

Biomarker validation

Prior to validation on the naïve cohort, MRM was used for quantitative confirmation of the FGA and UMOD cluster peptides in urine samples of the 28 infants used in the initial LC-MALDI discovery experiments. We then validated the urine samples from the 37 infants in the naïve cohort by the MRM method. Three of the candidate urine peptides (FGA1826, FGA1883 and FGA2659) were found to accurately discriminate the medical NEC from the surgical NEC groups in the discovery cohort (FGA1826, p value 7.25×10^{-4}; FGA1883, p value 2.13×10^{-6}; FGA2659, p value 1.49×10^{-6}; Figure 2D) and the naïve validation cohort (FGA1826, p value 1.07×10^{-2}; FGA1883, p value 1.33×10^{-6}; FGA2659, p value 2.45×10^{-5}; figure 2E). Among the three validated FGA peptides, FGA2659 was the marker with maximum abundance and peak discriminating capabilities between medical NEC and surgical NEC.

In order to gauge the clinical utility of the FGA peptide biomarker panel, the biomarker discovery cohort and the naïve biomarker validation cohort were assessed by iterative ROC testing (figure 3). The ROC curve for the biomarker discovery cohort revealed an AUC of 0.908. The ROC curve analysis of the naïve biomarker validation cohort was 0.858—indicative of a good prognostic test, however, this result was only marginally better then the clinical risk stratification model (AUC 0.817).

Ensemble algorithm combining clinical parameters and urine biomarkers

To improve the utility of the clinical parameter-based prognostic algorithm and biomarker panel, we combined the clinical and biomarker classifiers to develop an ensemble model for the prediction of NEC outcomes. We used the 64 subjects that had complete biomarker and clinical data sets (one infant's urine sample had been completely used in the prior biomarker experiments). Infants presenting with pneumoperitoneum on initial abdominal imaging (n=5) were considered to have surgical NEC and were assigned an arbitrarily high NEC ensemble outcome score. The clinical parameter-based prognostic algorithm, when used alone, resulted in significant overlap between the medical NEC and surgical NEC cohorts leaving 39.1% (total n=25/64; medical NEC, n=17/44; surgical NEC, n=8/20) in the indeterminate diagnosis group (figure 4A). The combination of the clinical parameter-based prognostic algorithm with the three FGA peptide biomarkers accurately predicted outcome for all infants in the medical NEC and surgical NEC groups (figure 4B).

DISCUSSION

There are currently no reliable prognostic instruments, clinical or biological, that accurately identify infants with progressive

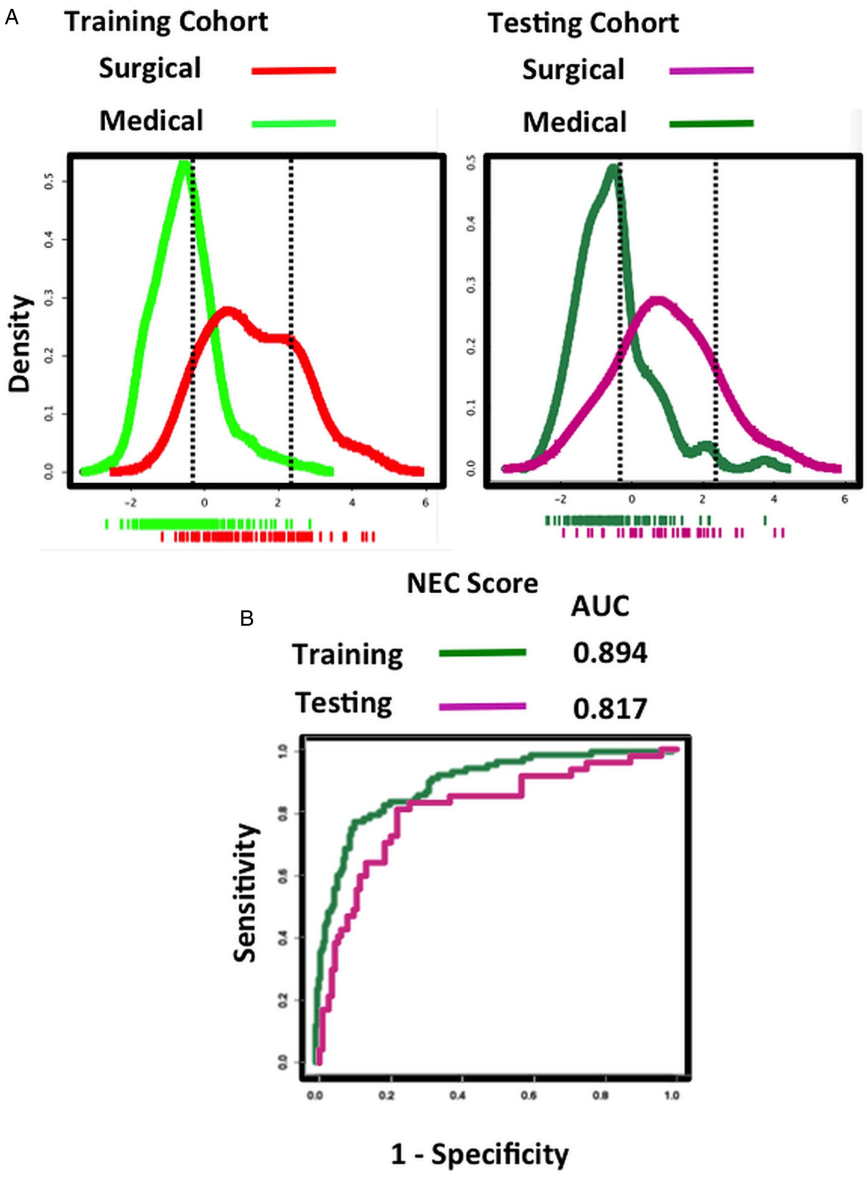

Figure 1 Clinical parameter-based diagnostic algorithm. (A)Density plots of medical necrotising enterocolitis (NEC) and surgical NEC infants' outcome scores based on clinical parameters. The area outside the dotted vertical lines represents prediction with 95% confidence, while the area between the lines represents the 'indeterminate' prediction. The percentage of infants with indeterminate predictions in the training and testing cohorts were 42.4% and 40.1%, respectively. (B) The performance of the linear discriminant analysis (LDA) model in outcome prediction by receiver-operator characteristic (ROC) area under the curve (AUC) analysis.

NEC prior to the development of irreversible intestinal damage and severe systemic illness. Bell's original staging criteria, with slight modification, are still widely used in the initial diagnosis of infants with NEC. Although Bell's criteria are useful at the time of diagnosis they have limited forecasting ability. We sought to define a novel ensemble prognostic algorithm by combining clinical data with novel urine biomarkers in order to accurately predict the presence of surgical NEC prior to overt clinical manifestation of disease. Risk-stratification by clinical parameters alone revealed an AUC of 0.817 by ROC analysis, while stratification by the biomarker panel alone revealed a slightly better AUC of 0.858. When combined, however, the ensemble algorithm fully discriminated between all infants with medical NEC and surgical NEC.

We have previously demonstrated that urine is a rich source of proteolytically cleaved proteins cleared from plasma by the kidneys and profiling analyses has proven highly informative for urogenital and systemic disease classification.[7–9 13 17 18] Importantly, the identified candidate peptide biomarkers in the current study have known biological functions supporting plausible roles in the pathophysiology of NEC. The described FGA peptides, in addition to being quantitatively validated and found to robustly predict disease progression in the ensemble model, contain overlapping sequences—suggesting that they reflect the activity of disease-related coagulation cascade proteases or their inhibitors.[19–25] The parent protein of our peptide biomarkers, fibrinogen A, represents the α chain of the fibrinogen protein. Fibrinogen is cleaved by thrombin during coagulation to form a fibrin thrombus. Thus it is conceivable that this peptide signature reflects the underlying advancing intravascular coagulation that is a distinct hallmark of progressive NEC. In addition, various cleavage products of fibrinogen have been reported to regulate cell adhesion, migration, vasoconstriction and inflammation as well as serve as mitogens for a variety of inflammatory cell types.[26–29]

Several recent publications have reported the discovery and validation of biomarkers that are either diagnostic of NEC or distinguish medical NEC from surgical NEC.[30] Significant measurable differences in platelet-activating factor,[30 31] C reactive protein,[32] inter-α inhibitor protein,[33] calprotectin, claudin,[34] and intestinal fatty acid-binding protein (I-FABP)[35] have all been reported as potential biomarkers for NEC, but none has had the necessary accuracy to be independently useful in the clinical setting.

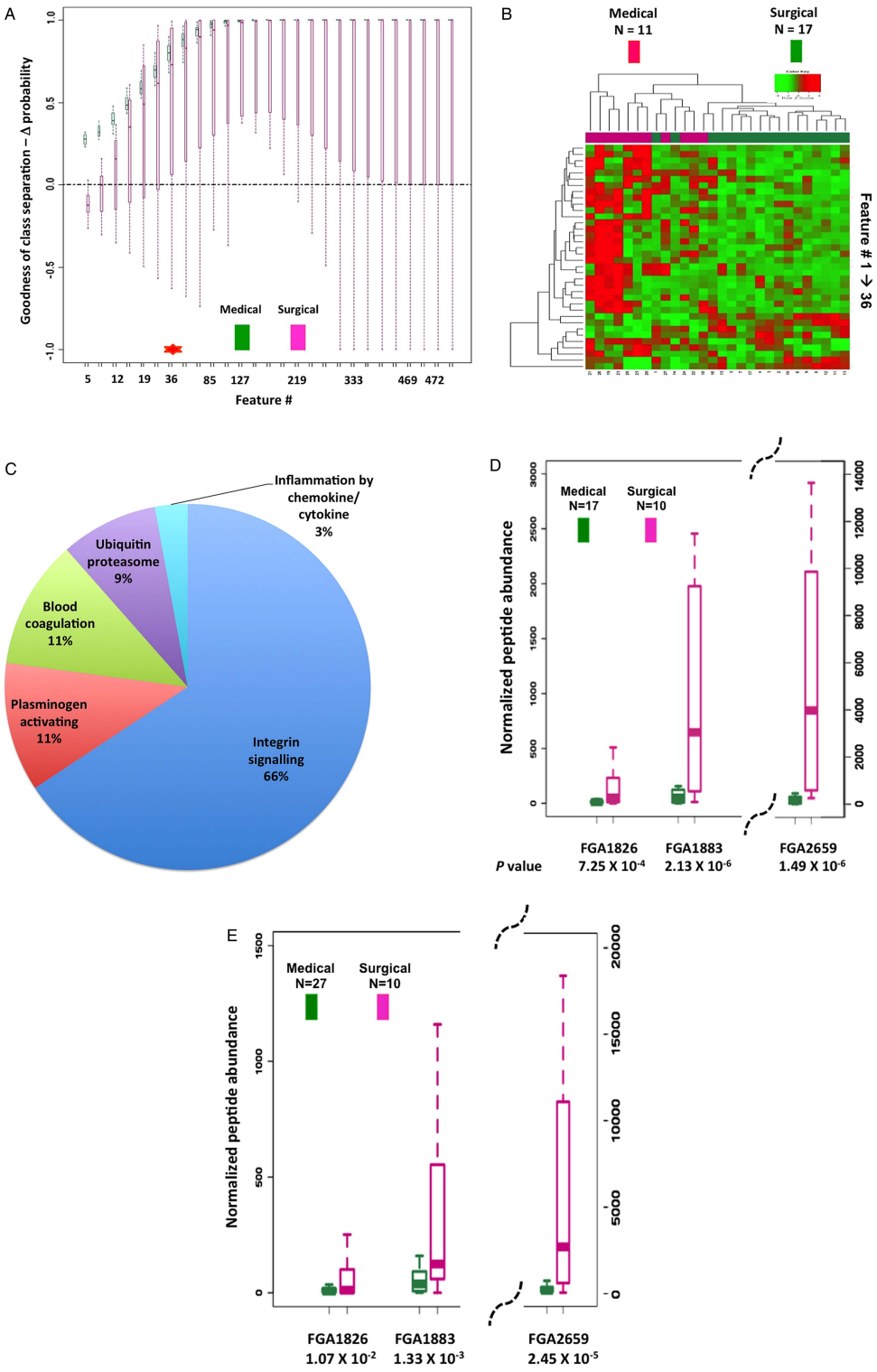

Figure 2 Biomarker discovery and validation. (A) Box and whisker plots of various feature sizes to distinguish the medical necrotising enterocolitis (NEC) and surgical NEC classes. Boxes contain 50% of values falling between the 25th and 75th percentiles; the horizontal line within the box represents the median value and the 'whisker' lines extend to the highest and lowest values. (B) Unsupervised hierarchical cluster analysis with heat map plotting demonstrating the association of NEC disease status with the abundance pattern of 36 peptide candidate biomarkers. (C) Summarised results of PANTHER database pathway analysis for the 36-peptide candidate biomarkers. (D) Validation of the peptide biomarkers by LC-MALDI in the biomarker discovery cohort (medical NEC, n=17; surgical NEC, n=10) and the (E) Biomarker validation cohort (medical NEC, n=27; surgical NEC, n=10). The whisker plots summarise the quantitative mass spec validation results in each of the depicted cohorts. Fisher's exact test indicates the significance of separation between the medical NEC and surgical NEC infants. FGA peptide sequences: FGA1826 DEAGSEADHEGTHSTKR; FGA1883 DEAGSEADHEGTHSTKRG; FGA2659 DEAGSEADHEGTHSTKRGHAKSRPV.

Furthermore, the majority of the prior studies included cases of advanced NEC (intestinal perforation) without attempt to risk stratify subjects with early disease or to exclude cases with advanced disease. This may lead to erroneous assumptions regarding the statistical performance and clinical utility of these biomarker studies.

Table 4 Necrotising enterocolitis (NEC) outcome predictive urine peptide biomarkers revealed by LC-MALDI urine peptidome profiling

	Relative abundance				
	Medical	Surgical	MW	Protein	Sequence
1	−0.04	0.046	1060.51	Q6ZUQ4	S.CKSPAQ@RRGG.S
2	−0.04	0.048	1217.58	OBFC2B	S.QP#NHTP#AGPP#GP.S
3	−0.02	0.024	1529.74	COL11A2	D.VGPMGP#PGPPGP#RGPAG.P
4	−0.1	0.119	1925.99	NBEAL2	Q.SVPASTGLGWGSGLVAPLQE.G
5	−0.02	0.02	1212.72	GRASP	P.P#ALPPPPPP#ARA.F
6	−0.31	0.363	2428.09	HUWE1	P.GP*SPGTGPGP*GP*GP*GPGPGPGPGPGPGPGP.G
7	−0.17	0.201	1752.83	COL1A2	A.GEKGPSGEAGTAGPP*GTP*GP.Q
8	−0.1	0.116	2088.85	HOXD3	P.GN@HHHGP#CDPHP#TYTDLSA.H
9	−0.02	0.026	1305.34	DSG4	L.YACDCDDNHM#C.L
10	−0.07	0.081	1143.28	KRTAP5-11	P.CCSSSGCGSFCC.Q
11	−0.09	0.104	1242.75	YI020	R.PKPSPPPPLILS.P
12	−0.3	0.356	2659.26	FGA	A.DEAGSEADHEGTHSTKRGHAKSRPV.R
13	−0.08	0.091	2560.18	FGA	A.DEAGSEADHEGTHSTKRGHAKSRP.V
14	0.033	−0.04	1680.96	UMOD	S.VIDQSRVLNLGPITR.K
15	0.098	−0.12	1912.06	UMOD	R.SGSVIDQSRVLNLGPITR.K

Relative abundance: PAM algorithm derived shrunken difference, derived by shrinking the class centroids toward the overall centroids after standardising by the within-class SD, for the 15 peptides between medical NEC and surgical NEC subjects.
MW, molecular weight.

A recent study by Reisinger *et al*[36] assessed the improved diagnostic accuracy of combining serum amyloid A and fecal calprotectin with intestinal fatty acid-binding protein. They found improved sensitivity and specificity when simultaneously assessing fecal calprotectin and I-FABP (sensitivity 94%, specificity 79%). Their findings support the notion that multiplexed analysis of various markers does indeed improve diagnostic instrument performance. They did not, however, differentiate between medical NEC and surgical NEC and the relatively small sample size requires further validation. Importantly, our ensemble model can accommodate additional biomarkers like fecal calprotectin and I-FABP as this will likely improve the performance of the instrument as we move into larger scale validation studies.

In addition to molecular biomarkers, physiological characteristics have been investigated as potential instruments for early diagnosis of NEC. Stone *et al*[37] studied the ability of advanced heart rate monitoring to provide early diagnosis of medical and surgical NEC. They were able to identify medical NEC approximately 6 h prior to clinical diagnosis and surgical NEC approximately 16 h prior to the clinical diagnosis. While intriguing, this technology does not appear to provide the lead time to diagnosis provided by our ensemble model. Certainly, the addition of heart rate characteristics into future iterations of ensemble modelling is an area for future exploration.

Ng *et al*[38] used a combined informatics and proteomics approach to construct a risk stratification model and to discover plasma protein biomarkers (proapolipoprotein CII and serum amyloid A) for infants with either NEC or sepsis. The study was not designed to distinguish NEC from sepsis or progressive NEC from non-progressive NEC and the overall number of confirmed cases of NEC was small. Their results were used to define a strategy for antibiotic usage in infants under suspicion of having either NEC or sepsis, but did not risk stratify infants at high risk for progressive NEC. In light of the current findings, we believe that the addition of novel biomarkers developed to

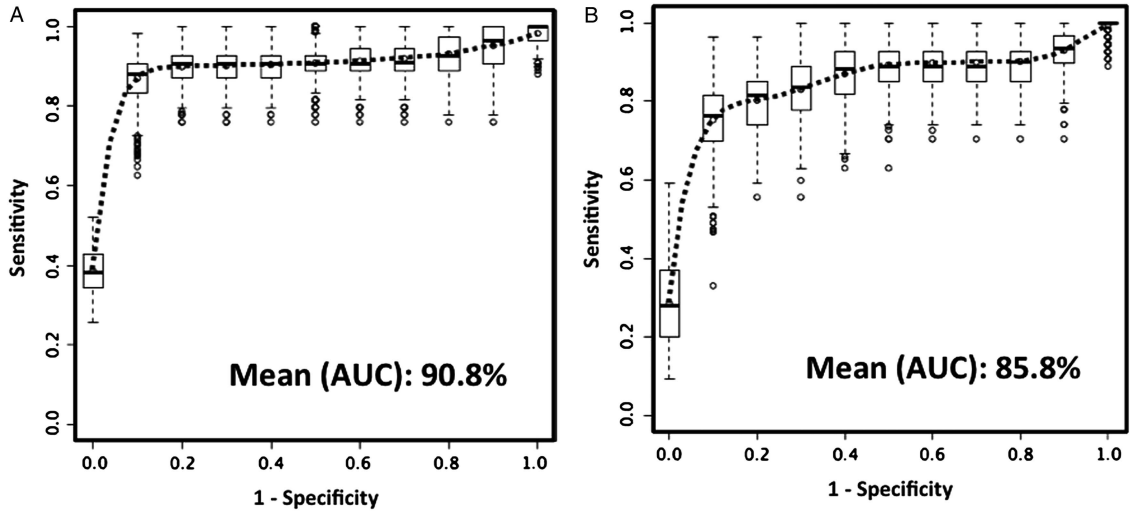

Figure 3 Receiver-operator characteristic (ROC) analysis and area under the curve (AUC) for the validated biomarkers. (A) Discovery cohort. (B) Validation cohort.

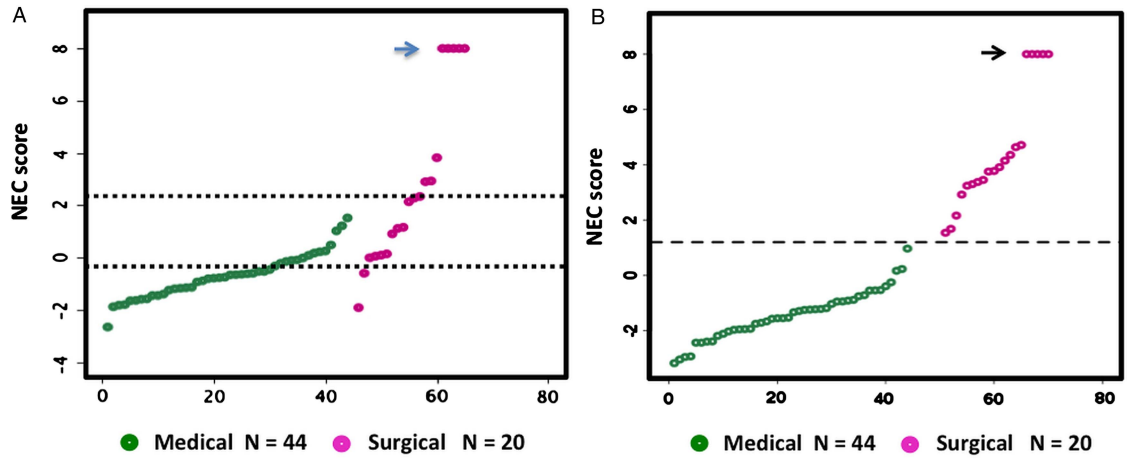

Figure 4 Performance of necrotising enterocolitis (NEC) Outcome Risk Stratification Algorithms. (A) Clinical parameter-based algorithm, 39% of all infants remain in the indeterminate group (n=25/64) represented by the area between the horizontal dotted lines. (B) Ensemble algorithm integrating clinical parameters with the fibrinogen (FGA) urine peptide biomarkers. The arrow indicates the five infants with pneumoperitoneum at presentation (assigned arbitrarily) with high prediction scores.

distinguish sepsis from NEC followed by risk stratification for NEC progression will lead to even greater utility in guiding patient management.

Despite the robust performance characteristics of our FGA ensemble model, there are several limitations. Although evidence of validation on a naïve subject cohort is provided, a larger prospective cohort validation study is warranted before clinical utility can be assessed. Given the high variability in timing of disease onset and progression of NEC, a longitudinal study to determine the changes in these candidate biomarkers relative to onset and course of disease will provide more evidence of potential utility.

Another important limitation is the technology presently employed to identify and quantify the urine peptides. From a technical perspective, urine peptidomics suffer from two major origins of variance, (1) analytical issues including mass spectrometric ion suppression and (2) biological issues including dilution of urine by different hydration states of the urine donors. Unfortunately, universal housekeeping peptides have not yet been identified in the urine peptidome to provide normalisation utility. From a more pragmatic perspective, MS is not currently a practical basis for a real-time clinical assay. Prior to widespread clinical utility, the development of a rapid urine fibrinogen peptide ELISA or other peptide identification assay will be necessary.

The clinical applicability of the prognostic instrument presented here is currently limited by the paucity of existing therapies for NEC. With further prospective validation of our model, however, immediate clinical utility as a triage instrument is a distinct possibility. High-risk infants could be rapidly transferred to high acuity facilities while transfer could potentially be avoided for those in the low-risk cohort. One could also envision decreased use of serial radiography or shorter duration of empirical antibiotic coverage for low-risk infants. The appropriateness of such practices, however, would require prospective clinical trials prior to widespread implementation.

Importantly, clinical risk stratification is a necessary first step in the development of novel treatment strategies. While it is true that few successful therapies have been developed for NEC, it also remains true that there has never before existed an accurate means to identify the high-risk population. As such, few clear metrics of success have been defined, previous studies have suffered from necessary design flaws, and many potential studies remain infeasible. As an example, early surgery is currently inconceivable given that roughly half of all infants with NEC will improve with non-operative management. An accurate risk-stratification instrument, however, would potentially enable the study of early operation for those at high-risk for disease progression. In the least, a prognostic instrument would provide a basis for the more accurate interpretation of the successes or failures of new therapies as they are developed.

Predictive models of disease progression are most useful when they are able to forecast an unforeseen event, signify a change in clinical trajectory or indicate the necessity for treatment. The findings presented here are a significant step towards these goals. We have shown that a combination of clinical parameters and biomarker analysis enables the early diagnosis of infants at risk for rapid disease progression while also accurately identifying those at low risk. While this model may presently be limited to use as a patient triage instrument, further refinement of the model has the potential to improve the care of infants at high risk for significant morbidity and mortality from NEC and identify those for whom novel prevention and treatment strategies may be useful.

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