Coagulation and Complement Protein Differences Between Septic and Uninfected Systemic Inflammatory Response Syndrome Patients

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Background: **Systemic inflammatory response syndrome (SIRS) represents a host response to various insults. Recent advances have demonstrated an interconnection between inflammation, complement, and coagulation. This experiment was designed to evaluate differences in plasma protein profiles between clinically identical patients: septic versus uninfected SIRS patients, prior to clinical diagnosis of infection.**

Methods: **Patients admitted to an intensive care unit of a major university, meeting two of four SIRS criteria were followed prospectively for development of sepsis. Plasma samples were collected daily and divided into two groups: a presceptic group that subsequently developed sepsis and a SIRS group that remained uninfected. Protein profiling was accomplished by three-dimensional liquid chromatography fractionation with electrospray ion trap mass spectrometry after immunodepletion of abundant proteins and a trypsin digest. Spectra peaks were identified using Agilent Technologies Spectrum Mill Workbench software. Relevance to biologic pathways was analyzed and statistical significance determined with DAVID 2.1 available at the National Institutes of Health.**

Results: **A total of 134 unique proteins were significantly different between groups. Thirty-two of these (23.5%) mapped to the complement and coagulation cascade (KEGG), 10 (7.5%) mapped to classic complement pathway; 11 (8.2%) mapped to complement pathway, and 8 (6.0%) mapped to lectin binding complement pathway (Biocarta). These pathways were all significantly ($p < 0.0001$) overrepresented in sepsis patients compared to SIRS-only patients.**

Conclusion: **Using novel mass spectrometry methodology, we were able to demonstrate differential protein profiles in septic versus uninfected SIRS patients prior to clinical diagnosis of sepsis.**

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The systemic inflammatory response syndrome (SIRS) represents the host response to numerous stimuli including trauma, burns, pancreatitis, transfusion reactions, and major surgery. Sepsis is defined as SIRS resulting from an infectious cause. Unfortunately, in the critically ill patient, sepsis is often difficult to diagnose as patients may already manifest SIRS from other illness. Approximately 70% of SIRS patients admitted to an intensive care unit (ICU) have a noninfectious cause.[1] However, the prompt diagnosis of sepsis is essential as early treatment is important for improving outcomes,[2,3] and sepsis remains the leading cause of death in noncoronary intensive care units.[4]

Standard diagnostic measures to identify infection include microbiologic cultures. Unfortunately, cultures can take >24 hours to obtain results and are neither sensitive nor specific. Other biologic markers have been studied as well. Procalcitonin has been considered a potential sepsis biomarker, and demonstrates prognostic capabilities. Wide spread use of procalcitonin in the ICU has been limited because of lack of specificity and variable sensitivity.[5] A recent meta-analysis confirmed the superiority of procalcitonin to C-reactive protein, but also identified its weakness as a diagnostic tool, suggesting it be used as a screening test with empiric antibiotics and further testing to accompany positive results.[6] Given the lack of a gold-standard molecular diagnosis for sepsis, there is an escalating search for biomarkers to help identify sepsis in the critically ill patient.

Recent developments in proteomics have allowed for analysis of complex protein fluids in greater detail than previously possible. Mass spectrometry has allowed for biomarker study and differentiation of complex samples in a multitude of diseases. Specifically the diagnosis of renal cell cancer,[7] breast cancer,[8] ovarian cancer,[9] and even the identification of intrauterine inflammation[10] have been suggested using mass spectrometry technologies.

This study was designed to evaluate differences in protein composition of plasma between critically ill SIRS patients who are becoming septic, as compared with that in critically ill SIRS patients who remain uninfected. Specifically, we hypothesized that the plasma protein composition of critically ill SIRS patients with sepsis would be different from plasma protein composition of phenotypically similar unin-

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fected patients manifesting SIRS. Furthermore, these differences will be detectable before the clinical diagnosis of sepsis.

METHODS

The Institutional Review Board of the University of Maryland School of Medicine approved this study.

As part of an ongoing study to characterize differences between sterile inflammation and sepsis, critically ill uninfected SIRS patients were prospectively evaluated for development of clinical sepsis. Patients over the age of 18 who were admitted to a trauma intensive care unit were screened. Trauma patients who met two of four standard SIRS criteria (Table 1)[11] and were clinically uninfected were enrolled. Exclusion criteria (Table 2) included potential immunocompromising states, administration of antibiotics for treatment, and extended prophylactic antibiotic use. Patients were divided into two groups: (1) uninfected SIRS, patients who remained uninfected for the course of the study; and (2) preseptic SIRS, SIRS patients who developed clinical sepsis during the course of the study. Sepsis diagnoses were based on the standard clinical criteria for SIRS and sepsis.[12,13]

Plasma was collected daily until ICU discharge (maximum 14 days) in the uninfected SIRS group. For the preseptic SIRS group, plasma was collected daily until the clinical diagnosis of sepsis then for a subsequent 3 days (maximum 17 days). Patient plasma was collected predominantly via a previously placed central venous catheter using a plasma preparation tube (BD Vacutainer, Franklin Lakes, NJ). Immediately after collection, samples were centrifuged at 1100× g for 20 minutes, and plasma was subsequently removed by pipetting and divided into 0.5-mL aliquots. Samples were stored frozen at -70°C until analyzed.

Table 1 SIRS criteria (must meet two of four for study entry)

Criteria	Description
Temperature	>38°C or <36°C
Respiratory status	Respiratory rate > 20, pCO_2 < 32 or mechanical ventilation
Heart rate	>90 bpm
White blood cell count	>12 k/mcl or >10% immature forms

Table 2 Exclusion criteria

Criteria
Known HIV positive at entry
Organ transplant recipient
Pharmacologic immunosuppression
Active or metastatic cancer
Recent chemo- or radiotherapy (within 8 weeks prior to enrollment)
Pregnancy
Spinal cord injuries having received steroids
Empiric antibiotic use upon entry
Investigational drug use within 30 days of enrollment
Prophylactic antibiotics longer than 48 hours duration

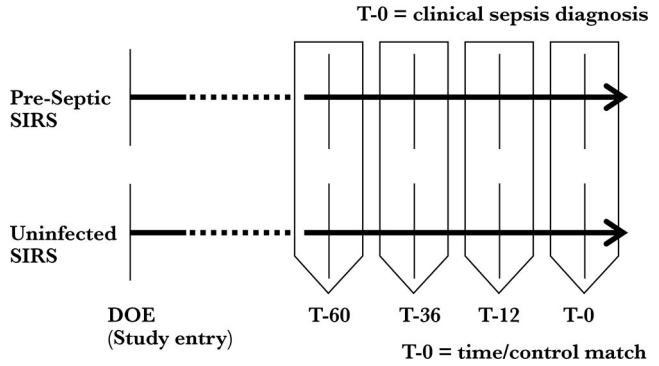

Fig. 1. *Time normalization scheme. Samples evaluated at T-60 (49 to 72 hours before T-0 time point); T-36 (25 to 48 hours prior to T-0); and T-12 (1 to 24 hours prior to T-0). T-0 was considered the time of clinical diagnosis of sepsis for the preseptic group or time-matched control for the uninfected SIRS group.*

To group patients by similar severity of disease and because preseptic patients converted to sepsis at varying time points after enrollment, all preseptic patients were retrospectively normalized using their clinical conversion to sepsis as the normalization point (T-0; Fig. 1). Clinical conversion time (T-0) was defined as the time a positive culture was obtained from an otherwise sterile location or direct visualization of perforated or necrotic bowel; *and* a clinical treatment (antibiotics and/or surgical procedure) was initiated for the infection as determined by majority consensus of an infectious disease attending, surgery attending, and a critical care attending. For the uninfected SIRS group, samples were time matched and T-0 normalized to clinically similar preseptic SIRS samples, based on demographic information, continued presence of SIRS, and elapsed time in the study. For both groups, samples were analyzed at four time points: DOE (day of study entry), samples drawn at study entry when both groups were uninfected; T-12, samples collected between 1 and 24 hours before the T-0 time point; T-36, samples collected 25 to 48 hours before T-0; and T-60, samples drawn 49 to 72 hours before T-0.

Protein profiling was performed in two experiments. Experiment 1 evaluated proteins differentially expressed at all time points tested between preseptic SIRS and uninfected SIRS in pooled plasma samples using a three-dimensional reverse phase/strong cation exchange/reverse phase liquid chromatography (LC^3) with electrospray ion trap mass spectrometry (MS^2), and spectrum counting for comparative quantitation[14] (performed by Mass Consortium Corporation, San Diego, CA). Briefly, plasma samples from 18 preseptic patients and 17 SIRS patients were pooled into 6 plasma pools (3 preseptic and 3 uninfected SIRS). Each individual pool was run at each time point. Samples were prepared by immunodepletion of abundant proteins (albumin, transferrin, haptoglobin, antitrypsin, immunoglobulin [Ig]G, and IgA) via Agilent Multiple Affinity Removal System (Agilent Technologies, Palo Alto, CA). Remaining protein was concentrated,

denatured in urea, reduced and alkylated, rediluted then digested twice with trypsin. The three-dimensional liquid chromatographic (LC^3) separation process previously described[15] was performed before loading. This process was necessary because traditional two-dimensional liquid chromatography (LC^2) is insufficient for these complex mixtures. Instead, the digest underwent a reverse-phase (RP) separation based on hydrophobicity, followed by strong-cation exchange (SCX) separation based on ion strength and then a third RP column was used to perform high resolution separation of the sample. Spectra peaks were identified and semiquantitated using Agilent Technologies Spectrum Mill MS Proteomics Workbench software (version 2.7, Agilent Technologies, Palo Alto, CA). MS/MS (MS^2) spectra were searched against the National Center for Biotechnology Information nonredundant protein database. The false-positive rate was estimated by autovalidating 4,294 spectra and 107 proteins by searching against a combined forward-reverse database. For proteins with at least two unique peptides, the false-positive rate was 2.8%. Spectrum counting was used for relative protein quantification. The total spectra numbers were normalized across all rounds and entries were removed if they had a distinct sum tag score less than 13. Sepsis-to-SIRS ratios were calculated using the normalized total spectra numbers. Where SIRS> sepsis, the ratio was calculated using 1/(sepsis/SIRS). If either number was zero, the entry was tagged SEPSIS+ or SIRS+ as appropriate. Discovered proteins were matched to Entrez gene identification.

In experiment 2, a slightly different procedure was performed. Electrospray ionization (ESI) LTQ-FTMS (Thermo Electron, Waltham MA) mass spectrometry profiling was run on pooled plasma on both groups collected at the T-12 time point. Large proteins were removed by centrifugal ultracentrifugation using a 30-kDa cutoff Centriplus ultrafilter (Millipore, Billerica, MA). This was followed by passing samples through an SCX and C18 column, before a single round of liquid chromatography. Eleven preseptic patients were compared with 10 uninfected SIRS patients. Peaks were identified using Agilent Technologies Spectrum Mill Workbench software. The data were normalized and ratios calculated identically to the first experiment. Proteins were matched to Entrez gene identifications.

To ascertain functional and relevant biologic pathways, the list of proteins identified as differential between the preseptic and uninfected SIRS groups was uploaded as their corresponding Entrez gene identifications to the Database for Annotation, Visualization, and Integrated Discovery version 2.1 (DAVID 2.1) software available from the National Institute of Allergy and Infectious Disease (http://NAID.abcc.ncifcrf.gov).[16,17] This allowed for annotation to biologic pathways. Statistical significance of pathways was analyzed by the Expression Analysis System Explorer (EASE) score. The EASE score, a

Table 3 Experiment 1 demographics

	Uninfected SIRS	Preseptic SIRS	*p* Value
Age (years)	45 ± 22	45 ± 28	ns
Sex (male:female)	72:28	70:30	ns
APACHE II	12.2 ± 5.1	14.6 ± 4.4	0.161
Injury Severity Score	29.2 ± 9.9	29.7 ± 9.6	ns
Trauma Injury Severity Score	0.84 ± 0.15	0.73 ± 0.30	0.19
Blunt mechanism (%)	100	72	
Closed head injury (n)	8	8	
Solid organ injury, liver or spleen (n)	5	8	
Hollow viscus injury (n)	0	4	
Pulmonary/cardiac injury (n)	6	10	
Major orthopedic injury, proximal long bone/pelvis (n)	7	10	

NS, not significant.

Table 4 Experiment 2 demographics

	Uninfected SIRS	Preseptic SIRS	*p* Value
Age (years)	44 ± 18	37 ± 16	ns
Sex (male:female)	70:30	90:10	
APACHE II score	12.2 ± 5.5	14.6 ± 4.5	0.32
Injury Severity Score	27.0 ± 9.1	30.1 ± 9.9	0.23
Trauma Injury Severity Score	0.80 ± 0.13	0.68 ± 0.35	0.48
Blunt mechanism (%)	100	45	
Closed head injury (n)	6	2	
Solid organ injury, liver or spleen (n)	0	5	
Hollow viscus injury (n)	0	4	
Pulmonary/cardiac injury (n)	3	4	
Major orthopedic injury, proximal long bone/pelvis (n)	4	2	

NS, not significant.

Table 5 **Experiment 1: differential proteins noted at T-60**

Symbol	Description	Directional Change (Sepsis/sirs)
AFM	Afamin	Increased
AHSG	Alpha-2-HS-glycoprotein	Decreased
APOA1	Apolipoprotein A-I	Decreased*
APOA2	Apolipoprotein A-II	Increased
APOA4	Apolipoprotein A-IV	Increased
APOB	Apolipoprotein B (including Ag(x) antigen)	Decreased
APOC3	apolipoprotein C-III	Decreased
APOH	Apolipoprotein H (beta-2-glycoprotein I)	Decreased
APOL1	Apolipoprotein L, 1	Increased
BCOR	BCL6 co-repressor	Decrease
BDP1	B double prime 1, subunit of RNA polymerase III transcription initiation factor IIIB	Decrease
C1QB	Complement component 1, q subcomponent, beta polypeptide	Increased
C1S	Complement component 1, s subcomponent	Increased
C3	Complement component 3	Decreased
C5	Complement component 5	Decreased
C8A	Complement component 8, alpha polypeptide	Decreased*
C9	Complement component 9	Decreased*
CD14	CD14 antigen	Increased*
CP	Ceruloplasmin (ferroxidase)	Decreased
CRP	C-reactive protein, pentraxin-related	Decreased
FGA	Fibrinogen alpha chain	Decreased*
FGB	Fibrinogen beta chain	Decreased*
FLNA	Filamin A, alpha (actin binding protein 280)	Increased
FN1	Fibronectin 1	Increased*
GC	Group-specific component (vitamin D binding protein)	Decreased*
HBB	Hemoglobin, beta	Decreased
HP	Haptoglobin	Decreased
HPX	Hemopexin	Decreased
HRG	Histidine-rich glycoprotein	Increased
IF	I factor (complement)	Increased
ITIH1	Inter-alpha (globulin) inhibitor H1	Increased*
ITIH2	Inter-alpha (globulin) inhibitor H2	Increased
ITIH4	Inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein)	Decreased
KLKB1	Kallikrein B, plasma (Fletcher factor) 1	Increased
KNG1	Kininogen 1	Decreased*
KRT1	Keratin 1 (epidermolytic hyperkeratosis)	Decreased
LGALS3BP	Lectin, galactoside-binding, soluble, 3 binding protein	Increased
LPA	Lipoprotein, Lp(a)	Decreased
LRG1	Leucine-rich alpha-2-glycoprotein 1	Decreased
MGC275	Hypothetical protein MGC27165	Increased
MYO18B	Myosin XVIIIB	Decreased
ORM1	Orosomucoid 1	Decrease*
PGLYR2	Peptidoglycan recognition protein 2	Decreased
QSCN6	Quiescin Q6	Decreased
RGS4	Regulator of G-protein signalling 4	Decreased
SAA1	Serum amyloid A1	Increased*
SERPINA1	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	Increased*
SERPINA3	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	Decreased
SERPINA6	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6	Increased
SERPINC1	Serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1	Increased
SERPIND1	Serine (or cysteine) proteinase inhibitor, clade D (heparin cofactor), member 1	Decreased
SERPING1	Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1	Decreased*
TRIP11	Thyroid hormone receptor interactor 11	Increased
VTN	Vitronectin (serum spreading factor, somatomedin B, complement S-protein)	Increased*

* Discordance between pools, predominant direction noted is listed.

Table 6 Experiment 1: differential proteins noted at T-36

Symbol	Description	Directional Change (Sepsis/sirs)
AFM	Afamin	Increased
AGT	Angiotensinogen (serine (or cysteine) proteinase inhibitor	Decreased
AHSG	Alpha-2-HS-glycoprotein	Decreased
ALMS1	Alstrom syndrome 1	Increased
APOA1	Apolipoprotein A-I	Increased
APOB	Apolipoprotein B (including Ag(x) antigen)	Decreased
APOE	Apolipoprotein E	Decreased
C2	Complement component 2	Decreased
C3	Complement component 3	Decreased
CP	Ceruloplasmin (ferroxidase)	Decreased
F2	Coagulation factor II (thrombin)	Decreased
FGB	Fibrinogen beta chain	Increased
FLJ10006	Hypothetical protein FLJ10006	Decreased
GC	Group-specific component (vitamin D binding protein)	Decreased
IF	I factor (complement)	Increased
IGFALS	Insulin-like growth factor binding protein, acid labile subunit	Decreased
ITIH1	Inter-alpha (globulin) inhibitor H1	Increased
KCTD7	Potassium channel tetramerisation domain containing 7	Decreased
KNG1	Kininogen 1	Decreased
LPA	Lipoprotein, Lp(a)	Increased
ORM1	Orosomucoid 1	Decreased
PDCD11	Programmed cell death 11	Increased
SERPINA1	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antitrypsin), member 1	Decreased
SERPINA3	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antitrypsin), member 3	Decreased
SERPINC1	Serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1	Decreased
SERPING1	Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1,	Increased
VTN	Vitronectin (serum spreading factor, somatomedin B, complement S-protein)	Increased

modification of the Fisher-exact test, allows for the ranking of biologic pathways associated with sets of genes and identifies functional categories over-represented in a gene list relative to its representation within the genome of a given species. Significant genes are mapped to known complement and coagulation cascade (KEGG)[18,19] and Biocarta[20] pathways.

RESULTS

For experiment 1, the patients were well matched for age and Acute Physiology and Chronic Health Evaluation (APACHE) II scores. Although APACHE II scores trended higher in the preseptic group, this difference was not significant (Table 3). The preseptic group did have a higher number of penetrating injuries and intra-abdominal injuries, but despite this, both Injury Severity Score (ISS) and Trauma Injury Severity Score (TRISS) were well matched between groups. Similar demographics were noted in experiment 2 (Table 4).

In experiment 1 at DOE, 55 proteins were differential between groups: 37 were semiquantitatively greater in the preseptic group, whereas 18 were decreased. At T-60, 54 unique proteins were noted to be differential between groups (Table 5), of which 22 were semiquantitatively greater in the sepsis group. At T-36, 27 unique proteins were noted to be differential between groups (Table 6), of which 10 were semiquantitatively greater in the sepsis group. At T-12, 38 unique proteins (Table 7) were noted to be differential between groups, of which 28 were semiquantitatively greater in the sepsis group. In all, accounting for proteins apparent in more than one time point, there were 71 unique proteins corresponding to unique Entrez gene identifications demonstrating significant differences between groups at the three time points before sepsis diagnosis excluding DOE.

In experiment 2, samples were run at T-12. We discovered 93 proteins corresponding to 93 unique gene identifications differential between groups at this time point (Table 8).

Of the 71 proteins from experiment 1 and 93 from experiment 2, 30 were identical between experiments (Table 9), 13 identical at the T-12 time point, as well as 17 found at T-12 in experiment 2 that were identical to proteins found at other time points in experiment 1. The union of unique proteins discovered by each experiment generated a total list of 134 unique proteins. To obtain an overall picture of the system changes occurring between septic and uninfected inflammation, this list was uploaded into DAVID 2.1 for analysis. Pathway analysis via EASE score demonstrated 32 of the 134 (23.5%, $p = 2.5 \times 10^{-42}$) mapped to the KEGG pathway: complement and coagulation cascade (Tables 10 and 11). Other major pathways (Biocarta) over-represented by our list include (all $p < 3 \times 10^{-8}$) classic complement pathway, 10 proteins (7.4%); complement pathway, 11 (8.1%); lectin-induced complement pathway, 8 (5.9%); intrinsic prothrombin activation pathway, 8 (5.9%); and the alternative complement pathway, 7 (5.1%). The fibrinolysis pathway (4 [2.9%]) and extrinsic prothrombin activation pathway (4 [2.9%]) both were significant at $p < 0.003$.

Table 7 Experimental 1: differential proteins noted at T-12

Symbol	Description	Directional Change (Sepsis/sirs)
AHSG	Alpha-2-HS-glycoprotein	Increased
APOA1	Apolipoprotein A-I	Increased*
APOA2	Apolipoprotein A-II	Increased
APOA4	Apolipoprotein A-IV	Increased*
APOC1	Apolipoprotein C-I	Increased
APOC3	Apolipoprotein C-III	Decreased
APOE	Apolipoprotein E	Decreased
APOH	Apolipoprotein H (beta-2-glycoprotein I)	Increased
BF	B-factor, properdin	Increased
C1S	Complement component 1, s subcomponent	Decreased
C3	Complement component 3	Increased
C4BPA	Complement component 4 binding protein, alpha	Decreased
C9	Complement component 9	Increased
CLU	Clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2)	Increased
F9	Coagulation factor IX (plasma thromboplastic component)	Increased
FN1	Fibronectin 1	Increased
GC	Group-specific component (vitamin D binding protein)	Increased*
HBB	Hemoglobin, beta	Increased
HPX	Hemopexin	Decreased
IF	I factor (complement)	Increased
ITIH1	Inter-alpha (globulin) inhibitor H1	Increased
ITIH2	Inter-alpha (globulin) inhibitor H2	Decreased
ITIH4	Inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein)	Increased
KLKB1	Kallikrein B, plasma (Fletcher factor) 1	Decreased
KNG1	Kininogen 1	Increased
LPA	Lipoprotein, Lp(a)	Decreased
LRG1	Leucine-rich alpha-2-glycoprotein 1	Increased*
ORM1	Orosomucoid 1	Increased*
QSCN6	Quiescin Q6	Increased
SAA1	Serum amyloid A1	Increased
SERPINA1	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	Increased
SERPINF2	Serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin,pigment epithelium derived factor), member 2	Decreased
SERPING1	Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1,	Increased
SMARCAD1	SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1	Increased
TF	Transferrin	Increased
TTN	Titin	Decreased
TTR	Transthyretin (prealbumin, amyloidosis type I)	Increased
VWF	Von Willibrand factor	Increased

* Discordance between pools, predominant direction noted is listed.

There were very few noncomplement/coagulation pathways statistically significantly represented within our group. Among the KEGG pathways, cell communication (10 proteins, 7.4%; $p < 0.0001$) and focal adhesion (9 proteins, 6.6%; $p = 0.012$) were significant. In the Biocarta subset, acute myocardial infarction (4 proteins, 2.9%; $p < 0.004$), cells and molecules involved in local inflammatory response (4 proteins, 2.9%; $p = 0.011$), and platelet amyloid precursor protein pathway (3 proteins, 2.2%; $p = 0.021$) were the only other significant pathways. As DAVID v2.1 analysis compares lists to the entire human genome, we were interested in knowing the significance of our data compared with the known protein composition of plasma. The coagulation and complement pathway contains 63 proteins as listed by DAVID v2.1. The number of proteins in human plasma has been estimated to be between 1,000 and 4,000. Using a conservative estimate of 1,275,[21] our 32 proteins related to complement and coagulation pathway still yield significance via Fisher's exact test of $p < 0.0001$.

DISCUSSION

Using novel mass spectrometry technology, we have identified differential proteins in the plasma proteome of critically ill septic patients compared with critically ill uninfected patients manifesting SIRS. Over 20% of the proteins demonstrating differences between these two groups are related to complement and coagulation.

The innate immune system is essential for the early recognition and defense against microbial invasion. Complement activation is considered an integral component of the

Table 8 Experiment 2: differential proteins noted at T-12

Symbol	Description	Directional Change (Sepsis/sirs)
A1BG	Alpha-1-B glycoprotein	Decreased
A2M	Alpha-2-macroglobulin	Decreased
ABLIM1	Actin binding LIM protein 1	Decreased
ACTA1	Actin, alpha 1, skeletal muscle	Increased
AGT	Angiotensinogen (serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 8)	Decreased
AHSG	Alpha-2-HS-glycoprotein	Decreased
ANK3	Ankyrin 3, node of Ranvier (ankyrin G)	Decreased
APCS	Amyloid P component, serum	Increased
APOA1	Apolipoprotein A-I	Decreased
APOA4	Apolipoprotein A-IV	Decreased
APOB	Apolipoprotein B (including Ag(x) antigen)	Decreased
APOC3	Apolipoprotein C-III	Decreased
APOL1	Apolipoprotein L, 1	Decreased
AZGP1	Alpha-2-glycoprotein 1, zinc	Decreased
B2M	Beta-2-microglobulin	Increased
BF	B-factor, properdin	Decreased
C1R	Complement component 1, r subcomponent	Decreased
C1S	Complement component 1, s subcomponent	Increased
C2	Complement component 2	Decreased
C4B	Complement component 4 beta	Increased
C5	Complement component 5	Decreased
C6	Complement component 6	Decreased
C7	Complement component 7	Decreased
C8A	Complement component 8, alpha polypeptide	Decreased
C8B	Complement component 8, beta polypeptide	Decreased
CDK5RA2	CDK5 regulatory subunit associated protein 2	Increased
CHGB	Chromogranin B (secretogranin 1)	Increased
CLU	Clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)	Decreased
COMP	Cartilage oligomeric matrix protein	Increased
CORO1A	Coronin, actin binding protein, 1A	Increased
CPN1	Carboxypeptidase N, polypeptide 1, 50kD	Increased
CUL1	Cullin 1	Decreased
DET1	De-etiolated homolog 1 (Arabidopsis)	Decreased
DSC1	Desmocollin 1	Increased
F13A1	Coagulation factor XIII, A1 polypeptide	Increased
F2	Coagulation factor II (thrombin)	Decreased
F5	Coagulation factor V (proaccelerin, labile factor)	Decreased
FGB	Fibrinogen beta chain	Increased
GOLGA1	Golgi autoantigen, golgin subfamily a, 1	Increased
GSN	Gelsolin (amyloidosis, Finnish type)	Decreased
HBA1	Hemoglobin, alpha 1	Decreased
HBB	Hemoglobin, beta	Decreased
HP	Haptoglobin	Decreased
HPX	Hemopexin	Decreased
HSPA5	Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	Increased
HUNK	Hormonally upregulated Neu-associated kinase	Decreased
IGFBP5	Insulin-like growth factor binding protein 5	Decreased
IGHG1	Immunoglobulin heavy constant gamma 1 (G1m marker)	Decreased
IGLV4–3	Immunoglobulin lambda variable 4–3	Increased
KIF5C	Kinesin family member 5C	Decreased
KNG1	Kininogen 1	Increased
KRT1	Keratin 1 (epidermolytic hyperkeratosis)	Increased
KRT9	Keratin 9 (epidermolytic palmoplantar keratoderma)	Decreased
KRT10	Keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris)	Decreased
LBP	Lipopolysaccharide binding protein	Increased
LGALS3BP	Lectin, galactoside-binding, soluble, 3 binding protein	Decreased
LRG1	Leucine-rich alpha-2-glycoprotein 1	Decreased
LUM	Lumican	Decreased
MMP14	Matrix metalloproteinase 14 (membrane-inserted)	Decreased

Table 8 Experiment 2: differential proteins noted at T-12 (continued)

Symbol	Description	Directional Change (Sepsis/sirs)
MYH4	Myosin, heavy polypeptide 4, skeletal muscle	Decreased
NEB	Nebulin	Increased
NUCB2	Nucleobindin 2	Increased
ORM2	Orosomucoid 2	Increased
PF4V1	Platelet factor 4 variant 1	Decreased
PIGR	Polymeric immunoglobulin receptor	Increased
PLG	Plasminogen	Decreased
PON1	Paraoxonase 1	Decreased
PPBP	Pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)	Increased
RBP4	Retinol binding protein 4, plasma	Decreased
RIMS1	Regulating synaptic membrane exocytosis 1	Decreased
RNF6	Ring finger protein (C3H2C3 type) 6	Increased
SAA1	Serum amyloid A1	Decreased
SEMA3D	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	Increased
SERPINA1	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	Decreased
SERPIND1	Serine (or cysteine) proteinase inhibitor, clade D (heparin cofactor), member 1	Decreased
SERPINF2	Serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2	Decreased
SERPING1	Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)	Decreased
SF3B1	Splicing factor 3b, subunit 1, 155kDa	Decreased
SPINK1	Serine protease inhibitor, Kazal type 1	Increased
SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	Increased
SPTB	Spectrin, beta, erythrocytic (includes spherocytosis, clinical type I)	Increased
SYNE1	Spectrin repeat containing, nuclear envelope 1	Increased
TAF4B	TAF4b RNA polymerase II, TATA box binding protein (TBP)-associated factor, 105kDa	Decreased
TBC1D1	TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1	Increased
TLN1	Talin 1	Decreased
TMSB4X	Thymosin, beta 4, X-linked	Decreased
TRIP11	Thyroid hormone receptor interactor 11	Decreased
TTR	Transthyretin (prealbumin, amyloidosis type I)	Decreased
UROC1	Urocanase domain containing 1	Decreased
VTN	Vitronectin (serum spreading factor, somatomedin B, complement S-protein)	Increased
VWF	Von Willebrand factor	Increased
ZFHX2	Zinc finger homeobox 2	Increased
ZYX	Zyxin	Decreased

innate immune system and the involvement of the complement system in our study is consistent with this concept. We have previously demonstrated activation of other components of innate immunity via gene expression profiling in similar patient populations.[22] Complement allows for elimination of invading cells and activation of the adaptive immune response by stimulating secretion of various cytokines. It has been suggested that the complement system could be a potential therapeutic target for sepsis.[23] Complement is activated by three distinct pathways. In the classical pathway, an antibody-antigen complex causes generation of C1q from C1. C1q binds to the Fc portion of the complex and activates C1r and C1s esterases. These cleave C2 and C4 forming C4b2a (C3 convertase). The alternative pathway does not involve antibodies. Instead, yeast zymogen, tissue-type plasminogen activator, and other substances such as some biomaterials allow formation of the alternative C3 convertase C3bBb. Finally, the Lectin pathway (mannose-binding pathway [MBL]) is activated by MBL binding to carbohydrate structures on invading pathogens. The serine proteases MASP-1 and MASP-2 then cleave C2 and C4 forming the classic C3 convertase. C3 convertase cleaves C5, and formation of C5b-9 membrane attack complex ensues. We have found elements of all three pathways to be differential between sepsis and sterile inflammation.

This study emphasizes the close association between sepsis and coagulation. The understanding of coagulation and sepsis at the molecular level has demonstrated the interconnected and intertwined nature of these processes. The profibrinolytic, antithrombotic, and anti-inflammatory drug, human recombinant activated protein C has been shown to reduce mortality from sepsis.[24] In these same septic patients, markers of coagulation and inflammation were related to disease severity.[25] Tissue factor is expressed in monocytes and macrophages in response to many inflammatory insults.[26–28] Additionally, proinflammatory cytokines cause increased expression of plasminogen activator

Table 9 Proteins common between experiments 1 and 2

Symbol	Description
AGT	Angiotensinogen (serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 8)
AHSG	Alpha-2-HS-glycoprotein
APOA1	Apolipoprotein A-I
APOA4	Apolipoprotein A-IV
APOB	Apolipoprotein B (including Ag(x) antigen)
APOC3	Apolipoprotein C-III
APOL1	Apolipoprotein L, 1
BF	B-factor, properdin (Alternate complement pathway)
C1s	Complement component 1, subunit s
C2	Complement component 2
C5	Complement component 5
C8A	Complement component 8, alpha polypeptide
CLU	Clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)
F2	Coagulation factor II (thrombin)
FGB	Fibrinogen beta chain
HBB	Hemoglobin, beta
HP	Haptoglobin
HPX	Hemopexin
KNG1	Kininogen 1
KRT1	Keratin 1 (epidermolytic hyperkeratosis)
LGALS3BP	Lectin, galactoside-binding, soluble, 3 binding protein
LRG1	Leucine-rich alpha-2-glycoprotein 1
SERPINA1	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
SERPIND1	Serine (or cysteine) proteinase inhibitor, clade D (heparin cofactor), member 1
SERPINF2	Serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2
SERPING1	Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)
SAA1	Serum amyloid A1
TTR	Transthyretin (prealbumin, amyloidosis type I)
VTN	Vitronectin (serum spreading factor, somatomedin B, complement S-protein)
VWF	von Willebrand factor

Table 10 Experiment 1 proteins annotated by DAVID 2.1 to KEGG pathway complement and coagulation cascade

Symbol	Description
BF	B-factor, properdin
F2	Coagulation factor II (thrombin)
F9	Coagulation factor IX (plasma thromboplastic component, Christmas disease, hemophilia B)
C1QB	Complement component 1, q subcomponent, beta polypeptide
C1S	Complement component 1, s subcomponent
C2	Complement component 2
C3	Complement component 3
C4BPA	Complement component 4 binding protein, alpha
C5	Complement component 5
C8A	Complement component 8, alpha polypeptide
C9	Complement component 9
FGA	Fibrinogen alpha chain
FGB	Fibrinogen beta chain
FGG	Fibrinogen gamma chain
IF	I factor (complement)
KLKB1	Kallikrein B, plasma (Fletcher factor) 1
KNG1	Kininogen 1
SERPINA1	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
SERPINC1	Serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1
SERPIND1	Serine (or cysteine) proteinase inhibitor, clade D (heparin co-factor), member 2
SERPINF2	Serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2
SERPING1	Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 2

inhibitor-1 and cause a decrease in protein C receptors. Although immune activation stimulates coagulation, the reverse is also true because various coagulation proteins such as thrombin, Factor Xa, and TF-VIIa complexes stimulate cytokine production. Activated platelets also secrete chemokines, promote neutrophil adherence, and, through CD-40, promote adhesion molecule expression on endothelium.

One strength of this study is the patient population and control groups. Instead of comparing sepsis to normal, healthy subjects, our preseptic group was compared with clinically similar critically ill patients manifesting SIRS. This allows for a better distinction between infected and uninfected SIRS in the ICU. Whereas evidence exists suggesting similar mechanisms for induction of inflammation via both infectious and noninfectious causes,[29–31] this study demonstrated coagulation and complement differences in these patient populations. Further, using two separate methods and different pools of plasma, a large group of proteins identical between sets was identified.

Understanding the complex interactions and changes in the plasma proteome of patients becoming septic could allow for better diagnostics and therapeutics. Many of the proteins identified do not have commercially available immunoassays and therefore new assays are being developed to verify and precisely quantify results obtained with this experiment. Future studies will test these proteins as potential biomarkers for sepsis. Understanding the complex systems events leading to sepsis may yield novel therapeutic targets. Further, since a subset of proteins demonstrated differential quantitation at study entry, it may be possible to stratify critically ill patients into various categories of risk of developing sepsis immediately at admission to the ICU.

One concern regarding this study is the calculated false-positive rate of 2.8% that may have resulted in up to four of our proteins considered false positives. However, even if all

Table 11 **Experiment 2 proteins annotated by DAVID 2.1 to KEGG pathway complement and coagulation cascade**

Symbol	Description
A2M	Alpha-2-macroglobulin
F2	Coagulation factor II (thrombin)
F5	Coagulation factor V (proaccelerin, labile factor)
F13A1	Coagulation factor XIII, A1 polypeptide
C1R	Complement component 1, r subcomponent
C1S	Complement component 1, s subcomponent
C2	Complement component 2
C4B	Complement component 4B
C5	Complement component 5
C6	Complement component 6
C7	Complement component 7
C8A	Complement component 8, alpha polypeptide
C8B	Complement component 8, beta polypeptide
FGB	Fibrinogen beta chain
KNG1	Kininogen 1
PLG	Plasminogen
SERPINA1	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
SERPIND1	Serine (or cysteine) proteinase inhibitor, clade D (heparin cofactor), member 1
SERPINF2	Serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2
SERPING1	Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)
VWF	von Willebrand factor

four map to coagulation and protein pathways, the list would still include 28 proteins or 20% of differentially expressed proteins as members of this group and the pathway would still be highly significant. Additionally, if a significant number of false-positive proteins were present, we would expect to see a larger variance in the categories of pathways identified. The fact that the vast majority revolve around similar themes of complement and coagulation suggests a low impact of the false-positive rate. For instance, we did not find any metabolism, endocrine, or cancer pathways. Another concern was a small set of discordant findings. As experiment 1 was run as three pools per time point, there were 15 proteins at T-60 and 5 at T-12 that demonstrated differences in directional changes between pools. Despite this, an overall picture of the direction and magnitude of change was still noted for these proteins.

A third concern would be the differences in mechanism of injury between groups. Among all proteins measured, there were 55 proteins significantly different at DOE. Forty-seven (85.4%) of these were also noted to be different at later time points preceding sepsis diagnosis. These 47 represent only 35% of the 134 unique proteins that were different before sepsis. Although these proteins may represent differences in mechanisms of injury, they may also suggest a protein-related predisposition to sepsis. This concept would potentially hold prognostic and/or predictive value and further study is indicated to ascertain if they are markers of a predisposition to sepsis. Specific to complement and coagulation proteins, there are 15 annotated proteins that were significant at day of study entry, in addition to the later study periods (Table 12). This group represented 46.9% of the 32 differentially expressed complement and coagulation proteins noted leading up to T-0. However, because the average time to T-0 was 7 days in both groups, and because APACHE II, ISS, and TRISS were well matched, those proteins demonstrating differences at the three time points before sepsis diagnosis but *not* at DOE, represent changes related to development of sepsis rather than differences in mechanism of injury.

Table 12 **Complement and coagulation proteins significant at DOE and at least one time point prior to sepsis diagnosis**

Symbol	Description
C1QB	Complement component 1, q subcomponent, beta polypeptide
C1S	Complement component 1, s subcomponent
C2	Complement component 2
C3	Complement component 3
C8A	Complement component 8, alpha polypeptide
C8B	Complement component 8, beta polypeptide
C9	Complement component 9
FGA	Fibrinogen alpha chain
FGB	Fibrinogen beta chain
FGG	Fibrinogen gamma chain
IF	I factor (complement)
KLKB1	Kallikrien B, plasma (Fletcher factor) 1
KNG1	Kininogen 1
SERPIND1	Serine (or cysteine) proteinase inhibitor, clade D (heparin cofactor), member 1
SERPINF2	Serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2

CONCLUSION

This study has identified specific plasma proteomic differences between critically ill SIRS patients who subsequently develop sepsis, and clinically similar SIRS patients who remained uninfected. These differences appear as early as 3 days before the clinical diagnosis of sepsis. Complement and coagulation proteins are statistically significantly overrepresented in this set. It is possible that a subset of these proteins may be useful as biomarkers for sepsis. Future study is warranted to evaluate these proteins for their potential predictive or diagnostic role.

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DISCUSSION

Dr. John T. Owings (Sacramento, California): Delayed treatment of infection leads to worse outcomes as published in *Chest* and a number of other journals. My disclaimers, although not financial, I'll list, and there are three.

First of all, this article agrees with the bias that I have voiced for the past 10 years, which is to say that the coagulation system and the inflammatory system are not two different systems, but rather one single system with different proteins that perform different functions.

The second one is that it is awfully hard to criticize very significantly an article that quotes your own articles.

And third, it hasn't escaped me that the recorder in this session is the senior author on the article.

Looking at the paper, there are several things that struck me: first of all, the inclusion criteria you could have included in this article simply by having a temperature of 38.1°C, with a white blood cell count of 12.1000. How many of our ICU patients don't have that?

The blood was very honestly reported as being predominately drawn from indwelling central venous catheters, or central catheters. It's well known that these catheters develop a fibrin sheath shortly after insertion. That then becomes proteinized. These proteins may reflect a very local environment and not the systemic environment. Nonetheless, it doesn't discount many of the findings. What do you think you would have found had you drawn the samples from, say, a peripheral stick, as we would with blood cultures typically, rather than a central line.

Next, how do you know which patients really were septic versus which patients had SIRS? Specifically, were all patients cultured the same, from the same sites, in the same way, or as is represented in the article—and I suspect is the

case—were certain patients identified as potentially having an infection, as we do clinically, cultures drawn, and then those cultures turn positive; and then those patients are placed into the Septic rather than the SIRS group? If this is the case, then truthfully, what this is a retrospective study of prospectively collected data.

If all patients were not cultured systematically, prospectively, why not? We all know that cultures, drawn from central venous catheters, have a tendency to grow bacteria, which doesn't necessarily mean that the patient is septic. It may simply mean that the catheter is colonized. Why is it colonized? Because it developed a fibrin sheath; the fibrin sheath got proteins to adhere to it, and the bacteria stick to the proteins. Were the cultures drawn from the central venous catheters?

The next question is, since this is the American Association for the Surgery of Trauma, I was interested to note that in comparing the groups, you compared APACHE scores, but not injury severity scores. And in experiment 2—it went by a little bit quickly—100% of the patients in the SIRS group were blunt trauma patients, whereas only 45% of the patients in the Septic group were blunt trauma patients. Why did you not evaluate the patients' traumatic injuries, since they were all trauma patients?

Finally, as much a comment as a question; I guess size matters. I will applaud you as having demonstrated to me the lowest p value I've ever read in an article, which was 2.5×10^{-42}; I guess size matters.

Finally, I think the authors have gone about something very novel and something very important, and that is to identify infection at its earliest point. With all of my concerns about, were some of the SIRS patients, in fact, septic? And were some of the septic patients, in fact, colonized?

I'm not sure they've done it, but they have raised great questions, and the best studies, I think, are the ones that are the most provocative and lead to future studies.

Dr. Krishnan Raghavendran (Buffalo, New York): As I understand proteomics, one of the key integral parts of proteomics is bioinformatics. I think that your statistical calculation is far from accurate if you want to do proteomic analysis.

Just simply taking two grooves and figuring out which ones are overlapping is clearly, in my opinion, not the right way to do it, because that way, you're only looking at proteins that are expressed in one versus the other.

How do you not know whether in one category, they were really increased; and in the other category, they were really reduced? So how do you not know the significance of that?

And then any form of proteomic analysis, especially in humans, is the essence of cytokines that can be produced. So why did you not employ features such as LPS stimulation to figure out the nature of intercellular cytokines that were produced?

Dr. Mitchell J. Cohen (San Francisco, California): The first question is why were the samples not matched for injury? It seems to me that the date of entry, the differences in the protein expression or activation, date of entry should be primarily from differences in injury and not necessarily differences in their septic condition.

I know that you were matched by APACHE scores, but why not by type of injury or ISS? My second question is, what's next? I understand you pooled the samples for convenience and technology, but I'm wondering if you have run samples on specific individual patients and looked at the differences in patients based on their injury or their individual physiology?

Lastly, I'm curious if you have done any probabilistic modeling or monitoring to know whether or not you can actually predict whether patients will get septic based on early measurements of these tests?

Dr. Matthew Lissauer (Baltimore, Maryland): Dr. Owings, in terms of drawing blood for cultures, all blood cultures were drawn peripherally as that is our protocol. As for blood drawn for study purposes, the majority in both groups were drawn from central lines. Might that affect the number of complement and coagulation proteins identified? I suppose that's possible. Despite a waste being drawn, it still might influence our results. However, the majority of blood was drawn centrally in both groups, and since we were looking at differences between groups, any impact of central line biofilms should have been observed in both groups equally. As we go forward and move into more prospective work we will take that into account.

In terms of which patients were septic and which had SIRS, all patients were not cultured the same. This was, as you mentioned, a retrospective look at prospectively gathered data. Basically, we retrospectively identified a time point when the majority of 3 physicians said, "This patient is septic, start an antibiotic". We took the time of sepsis as the time they had the definable, positive culture taken. We studied plasma samples from the four time points prior to this.

If all patients were not cultured prospectively, why not? That had to do with IRB approval. We were already drawing close to our allowed limit of blood for other study purposes, so unfortunately we couldn't do that. As we gather data and are able to design more prospective studies, this is certainly something we could incorporate into their design to eliminate confounders

As for the question of why such a low p value, when looking at the EASE modification of Fisher's exact test, DAVID 2.0 software compares your "buckets" of genes to the whole genome. If you go back and look at the "buckets" of proteins that are in plasma, and that has been published, there are anywhere from 1,000 to 4,000 known plasma proteins. Using a conservative estimate of 1,275 (published several years ago), performing Fisher's exact test with the number of complement and coagulation proteins found in comparison to the ratio of known complement and coagulation proteins in plasma, yields a p value of about $10^{-5 \text{ or } 6}$.

Dr. Raghavendran's questions of detailed biostatistical analysis/bioinformatics and LPS stimulation are interesting. This is our initial evaluation of this data. We really just wanted to determine if we could identify proteins as being different between groups. With further analysis we expect to see trends over time with some proteins starting out high in one group and decreasing over time as a patient develops sepsis and vice versa.

In terms of LPS stimulation and other basic experiments, that is real bedside back to bench translational research and that is where we want to go in the future. This was initial, clinical, translational work: bringing the bench to the bedside. Knowing what we now know, we can go back to the lab with some of these proteins, and really get a good handle on the biologic changes that take place in sepsis.

In regards to Dr. Cohen's comments about why did we match with APACHE scores and not ISS or other injury scales, the focus of this study was critically ill patients and therefore we think that controlling for physiologic derangement by APACHE was more appropriate. Controlling for anatomic derangement may be of value but was not the focus of this study. Of note when we have looked at ISS in our database, it is similar between preseptic and uninfected patients overall.

In terms of why we did not match for traumatic injuries or evaluate traumatic injuries. In experiment 2 there was a difference in mechanism of injury, and that certainly may have affected our results. The extent of impact would be speculative but since no difference was noted in experiment 1, we doubt that mechanism is a significant confounder, but will look into the question.

What's next? Again this was our initial attempt at looking at differences between critically ill SIRS patients becoming, but not yet clinically diagnosed with sepsis compared with those that remain uninfected. We identified a group of proteins that may be differential between groups. Some of them are not common proteins that you usually find or that have commercially available assays. We're developing assays for them. With that technology we'll be better able to take a look at a larger number of individual patients as opposed to groups and see if we can determine differences between groups.

Finally, in terms of probabilistic modeling or predictive value, we have not done this yet with our proteomics. However, for our genetic data, we have created some models. We are able to predict with sensitivities and specificities in the mid to high 80s whether a patient will develop sepsis, but that work is still early.