Continuous evaluation of changes in the serum proteome from early to late stages of sepsis caused by *Klebsiella pneumoniae*

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Abstract. Serum protein profiles of patients with bacterial sepsis from the day of diagnosis until recovery/mortality were compared from early to late stages in response to severe sepsis using two dimensional electrophoresis. The proteins exhibiting changes during the course of sepsis (20-28 day mortality) were selected and identified by matrix-assisted laser desorption ionization-time of flight-tandem mass spectrometry. Among the proteins identified, haptoglobin (Hp), transthyretin (TTR), orosomucoid 1/α1 acid glycoprotein (ORM1), α1 antitrypsin (A1AT), serum amyloid A (SAA) and S100A9 exhibited differential expression patterns between survivors (S; n=6) and non-survivors (NS; n=6), particularly during the early stages of sepsis. Expression factors (EFs), taken as the ratio between the NS and S during early stages, showed ratios of Hp, 0.39 (P≤0.012); TTR, 3.96 (P≤0.03); ORM1, 0.69 (P≤0.79); A1AT, 0.92 (P≤0.87) and SAA, 0.69 (P≤0.01). S100A9, an acute phase protein, exhibited an EF ratio of 1.68 (P≤0.004) during the end stages of sepsis. A delayed rise in levels was observed in Hp, A1AT, ORM1, S100A9 and SAA, whereas TTR levels increased during the early stages of sepsis in NS. Analysis of inflammatory responses in the early stages of sepsis revealed increased mRNA expression in leukocytes of interleukin (IL)-6 (EF, 2.50), IL-10 (EF, 1.70) and prepronociceptin (EF, 1.6), which is a precursor for nociceptin in NS compared with S, and higher Toll-like receptor-4 (EF, 0.30) levels in S compared with NS. Therefore, a weaker acute phase response in the early stages of sepsis in NS, combined with an inefficient inflammatory response, may contribute to sepsis mortality.

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Abbreviations: APR, acute phase response; A1AT, α 1 antitrypsin; CRP, C reactive protein; CNTF, ciliary neurotrophic factor; DAMPs, damage associated molecular patterns; GO, gene ontology; Hp, haptoglobin; HBB, hemoglobin β subunit; IL, interleukin; LPS, lipolysaccharide; MAP, kinase-mitogen activated protein kinase; NS, non-survivor; ORM 1, orosomucoid 1; PAMPs, pathogen associated molecular patterns; PCT, procalcitonin; S, survivor; SAA, serum amyloid A; SOFA, sequential organ failure assessment score; TLRs, Toll-like receptors; TNF, tumour necrosis factor; TTR, transthyretin

Key words: bacterial sepsis, acute phase proteins, two dimensional gel electrophoresis, serum proteome

Introduction

The inflammatory response to a variety of systemic infections results in sepsis in susceptible individuals, wherein the mortality rate is an alarming 30-45%. Sepsis constitutes 17% of all admissions to the intensive care unit, of which 45% of admissions end in fatality (1). Severe sepsis takes more lives than cardiovascular disease, breast, colon/rectal, head and neck, throat and prostate cancer combined, and the incidence of sepsis is rising by 1.5-8% annually (2-4), despite advancements in critical care support and equipment. While very little is known about the genetic susceptibility of an individual, the risk of sepsis increases due to various immunosuppressive procedures.

Several novel approaches in sepsis prophylaxis and treatment are currently in progress (4). Certain clinical trials have provided contrasting results. For example, when patients with sepsis were treated with two different monoclonal antibodies against endotoxin-HA-1A, a human antibody and E5, a murine antibody, no change in mortality was observed in patients with gram negative bacteremia (5). Animal models have also provided conflicting results when compared with human studies (6). Therefore, using an animal sepsis model may not provide conclusive evidence for human application. Immunological response to Gram-negative bacterial lipopolysaccharide (LPS) predominantly involves their interaction with Toll-like receptors (TLRs) and cluster of differentiation (CD)14 receptors present on monocytes and macrophages, which initiates the production of pro-inflammatory mediators, including interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)-α. LPS also induces the production of acute phase response proteins by the liver. Among many known sepsis markers, serum procalcitonin (PCT) is currently the only US Food and Drug Administration approved biomarker for the diagnosis, and as an indicator, of

the progression of sepsis, though other acute phase reactants, including C reactive protein (CRP) and serum amyloid A (SAA) protein, are also in use. Several biomarkers are known to be elevated in sepsis (7), although the exact biochemical function and etiology of their overexpression remain unknown. Host immunological response to sepsis is nuanced, and varies in both innate and adaptive responses, which makes diagnosis and therapy a challenge for individuals at risk of mortality.

The diagnosis of sepsis and evaluation of its severity is complicated by the highly variable and non-specific nature of the signs and symptoms of sepsis (8). Early diagnosis and prediction of the severity of sepsis is very important, thereby increasing the possibility of starting timely and specific treatment (9,10). Previous studies have shown gender-based variation in the pattern of expression of acute phase proteins and sepsis-associated mortality (11), wherein mutually opposing observations have been made, which make adjustments with concurrent data while generalizing observations (12). A previous study involving elderly patients revealed that sepsis mortality was independent of gender; however, this was correlated with elevated 17 β-estradiol in both genders, with elevated progesterone in males and elevated testosterone in females (13). An increased risk of acquiring sepsis in surgery patients with higher TNF-α levels due to polymorphism in the *Nco*I region of the TNFB gene has been reported (14,15). However, a generalization in this regard requires study of a larger cohort. Recent advances in understanding sepsis involve various sepsis models to diagnose susceptibility towards sepsis, however, a clear correlation requires a broader and deeper analysis of sepsis response proteins. To address the issue, Kalenka *et al* (16) analyzed the serum proteome of sepsis patients and successfully identified differences between the proteome of survivors (S) and non-survivors (NS) at the end of 28 days from the onset of sepsis (16). Similarly, Su *et al* (17) studied urinary proteomics of sepsis patients during the 28 days from the onset of sepsis (17). These studies have made significant contributions to the understanding of serum protein dynamics to assess the differential changes associated with S and NS of sepsis. The present study is a prospective observational longitudinal study, where serum proteome dynamics from early until late stages of sepsis were analyzed in S and NS, from the onset of sepsis as indicated by PCT levels.

Since sepsis has a higher incidence in males, and females appear to differ in responses due to hormonal variations, the present study used adult human male samples for homogeneity. The goal of the present study was to target differentially expressed proteins while comparing S and NS, which may be useful, particularly in the early stages to devise strategies to improve chances of patient survival. The present study focused on serum proteome profiles at different phases of sepsis in Indian adult male patients suffering with bacterial sepsis, particularly *K. pneumoniae*, to eliminate further possibility of heterogeneity in sampling, which may assist with understanding changes in serum acute phase proteins under given conditions, and can be later used to monitor and devise methods of patient-specific sepsis management.

Materials and methods

Patients and samples. Blood samples from adult male patients (n=12; S and NS =6 each) diagnosed with sepsis were procured from Global Hospitals (Lakdi-ka-pul Hyderabad, India; Table I). The patients were carefully monitored up until mortality at day 20-28, and samples were collected daily from the day of clinical diagnosis (onset) until recovery, in the case of S, and 24 h prior to mortality in the case of NS. Criteria for selection of male patients showing signs of severe sepsis or septic shock (endotoxemia) were based on patient serum PCT levels and acute physiology and chronic health evaluation II (APACHE II) scores (Table I). APACHE II is a severity-of-disease classification system (18), one of several ICU scoring systems. It is applied within 24 h of the admission of a patient to an intensive care unit: An integer score from 0 to 71 is computed based on several measurements; higher scores correspond to more severe disease and a higher risk of death. The APACHE II scoring system has been widely accepted as a measure of illness severity; it has been demonstrated to accurately stratify risk of death in a wide range of disease states, and in different clinical settings (19). Blood samples from healthy males (n=6) were collected with their consent as reference controls. Serum was isolated from blood samples of both patients and healthy controls for further analysis. Whole blood was collected separately from the identical male patients (S=6; NS=6) at early stages (within 24 h of sepsis diagnosis) in EDTA-K3 containing tubes for RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Exclusion criteria for sample selection were being <18-years-old and >75-years-old, patients who were lost at follow-up, patients with previous medical history of anti-inflammatory drug treatment, chemotherapy and glucocorticoid therapy. Burns patients, subjects with liver diseases, cardiovascular diseases and organ transplant recipients were not enrolled. Samples from each individual patient were collected with the informed consent of the patient or family, and the present study was approved by the institutional ethical committee.

Two dimensional electrophoresis. Serum samples for each day from the onset of sepsis until the recovery/death of each patient (n=6) and individual controls (n=6) were subjected to Albumin depletion, according to the manufacturer's protocol (Aurum™ Affi-Gel® Blue mini kits and columns; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Complete albumin depletion was confirmed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The albumin-depleted elutes were subsequently diluted with ice-cold acetone and subjected to acetone precipitation at 15,000 x g at 4˚C for 10 min. The pellets obtained were washed with 1 ml acetone and allowed to air-dry. The pellets were subsequently resuspended in 75 μl rehydration sample buffer, containing 4 M urea, 2% CHAPS, 1 mM dithiothreitol, 0.2% Biolyte, 3/10 ampholytes (Bio-Rad laboratories, Inc.) and traces of Bromophenol blue dye. Once the pellets were completely dissolved in rehydration sample buffer, the total protein content in resuspended sample was estimated using Bradford protein assay (Sigma-Aldrich, St. Louis, MO, USA). A total of 500 μg protein was applied to 11 cm (pH 3-10) immobilized pH gradient (IPG) strips (Bio-Rad Laboratories, Inc.) and isoelectric focusing was performed using a Protean IEF unit, according to the manufacturers protocol (Bio-Rad Laboratories, Inc.). The IPG strips were subsequently separated

Table I. Clinical characteristics of sepsis subjects.

Characteristic	Patient details	
	Survivor (n=6)	Non-survivor (n=6)
Age	50±2	71±2
Gender	Male	Male
Median white blood cell count/mm^3	14,380	1,850
Median serum procalcitonin	3.99±2	2.58±2
APACHE II (median)	26	30
Pathogens detected in culture (median)	K.p	K.p

APACHE II, acute physiology and chronic health evaluation II; K.p, *Klebsiella pneumoniae*; n, number of patients.

by 9-14% gradient SDS-PAGE at 16 mA for the stacking gel and 24 mA for the resolving gel. Following electrophoresis, the gels were fixed in a solution of 50% methanol and 10% glacial acetic acid for 1 h and were subsequently stained with colloidal Coomassie blue stain for image analysis.

Image analysis. The gel images were analyzed using Image Master 2D Platinum software (version 7.0; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Control gels of healthy subjects (n=6) were analyzed individually and normalized to be used as the reference gel. The daily sample for S and NS was analyzed in duplicate to identify spots with percentage volume variation. Duplicates of each day were combined to give a single representative gel and analyzed again to increase the reproducibility and reduce the error rate of analysis. The spots were compared for percentage volume variations.

In-gel trypsin digestion. The spots of interest were excised and washed with a 1:1 ratio of 50 mM ammonium bicarbonate and acetonitrile for 15 min. Following two separate washes with ammonium bicarbonate and acetonitrile, a final wash was performed with acetonitrile until the gel pieces were opaque. The acetonitrile was discarded and the gel pieces were vacuum-dried. Diluted trypsin was added to the gel pieces and incubated for 1 h at room temperature. The excess trypsin was removed and the pieces were incubated overnight with 25 mM ammonium bicarbonate at 37˚C. The digested extract was collected and vacuum centrifuged to concentrate the extract, which was subsequently used for matrix-assisted laser desorption ionization-time of flight (MALDI-TOF; Bruker Daltonics, Leipzig, Germany) analysis.

MALDI-TOF analysis. An α-cyano-4-hydroxycinnamic acid (HCCA) matrix (5 mg/ml) was prepared in 70% acetonitrile and 30% 0.1% trifluoroacetic acid. The trypsin-digested extract was subsequently mixed with HCCA matrix in a 1:1 ratio and ~2 μl matrix-sample mix was spotted onto an anchor chip and ground steel plate (Bruker Daltonics). Once dried, the plates were loaded onto a MALDI TOF-mass spectrometer (MS; Bruker Daltonics) at the Central Facilities for Research and Development (Osmania University, Hyderabad, India). Spectra were obtained in the reflectron mode (mass range, 500-3,000 Da; 20 keV accelerating voltage; averaging 500 laser shots/spectrum) using a Bruker Autoflex III MALDI-TOF/TOF spectrometer (Bruker Daltonics). The spectra were analyzed with Flex Analysis software (version 3.3; Bruker Daltonics) and Biotools software (version 3.2; Bruker Daltonics), with the following parameters: Signal-to-noise threshold, 6; mass exclusion tolerance, 0.75 m/z; maximal number of peaks, 100; quality factor threshold, 50; monoisotopic peaks (Adduct: H). Matrix and/or auto-proteolytic trypsin peaks, or known contaminant ions were excluded. Bioinformatics data mining was performed using the Mascot platform (http://www.matrixscience.com). The resulting peptide mass lists were queried in the Swiss Port 2013_02 database (539,165 sequences; 191,456,931 residues). The following criteria were used for search parameters: Taxonomy, *Homo sapiens* (human); significant protein Molecular Weight Search score at P<0.05, 1 missed cleavage site allowed; 1+peptide charges allowed; trypsin as enzyme; 80-100 ppm as precursor tolerance; carboxymethylation of Cys as global modification and oxidation of methionine as variable modification. The protein score was calculated as -10xLog (P), where P is the probability that the observed match is a random event. A protein score of ≥56 was considered statistically significant (P<0.05). Further analysis and function-based classification of the identified proteins were performed using the protein centre software version 3.10 (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Statistical analysis. Comparisons between groups (S and NS sepsis patients) were performed using either a paired Student's t-test and/or a Mann Whitney U test, using GraphPad Prism (version 6.05; GraphPad Software,. Inc., San Diego, CA, USA). The expression factors (EFs) are expressed as the mean ± standard error of the mean. P≤0.05 was considered to indicate a statistically significant difference.

RT-qPCR analysis. Blood samples from sepsis patients were obtained and the total RNA was extracted using Total RNA spin columns (Yeastern Biotech, Co., Ltd., Taipei, Taiwan) and treated with RNase-free DNase (Macherey-Nagel, Inc., Düren, Germany). A total of ~5 μg RNA was reverse-transcribed using oligo (dT) primers and reverse transcriptase (Thermo Fisher Scientific, Inc.). qPCR was performed on the cDNA samples

Table II. List of primer sequences for analyzing mRNA levels in patients with sepsis.

Gene	Right primer (5'-3')	Left primer (5'-3')
Haptoglobin	CATAGCCATGTGCAATCTCG	AGAGGCAAGACCAACCAAGA
S100A9	TCAGCATGATGAACTCCTCG	GGAATTCAAAGAGCTGGTGC
Transthyretin	AGCCGTGGTGGAATAGGAG	CTTACTGGAAGGCACTTGGC
Serum amyloid A	CCCTTTTGGCAGCATCATAG	AGCCGAAGCTTCTTTTCGTT
α 1 antitrypsin	ACGAGACAGAAGACGGCATT	ATATTCACCAGCAGCCTCCC
Orosomucoid 1	CCTCCTCCTGTTTCCTCTCC	AGACGACCAAGGAGCAACTG
Interleukin 6	CTGCAGCCACTGGTTCTGT	CCAGAGCTGTGCAGATGAGT
Toll-like receptor 4	GCCTCAGGGGATTAAAGCTC	GCCTCAGGGGATTAAAGCTC
Interleukin 10	GCCACCCTGATGTCTCAGTT	GTGGAGCAGGTGAAGAATGC
Prepronociceptin	GAGACTGAGCAGCAGCAGGT	TATGCTGGTGTGGCTGAGAA

(2 μl) using Faststart universal SYBR Green master (Roche Diagnostics, Indianapolis, IN, USA) with 10 pmol forward and reverse primers of six protein genes obtained employing Primer Depot (Table II). Melting temperatures were set as per primer sets used with rest of the PCR conditions. Gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method, where β-actin gene was used as the reference housekeeping gene.

Results

Identification of differentially expressed proteins in S and NS of sepsis. Albumin-depleted serum samples from sepsis patients were separated by 2D gel electrophoresis and 2D gel image analysis was performed (Fig. 1). Protein spots representing specific proteins exhibiting an increased or decreased percentage volume and intensity were matched to the corresponding spot in the reference gel (control serum). The proteins exhibiting differential volume percentage with respect to patients with sepsis were then matched between S and NS from the day of onset until recovery/mortality. Approximately 300 spots were analyzed in each gel. The analysis resulted in the identification of 30 differentially expressed spots between S and NS. Identification of the spots by MALDI-TOF demonstrated 12 spots with a significant MS/MS score (Table III). Since normalized relative volumes of a spot (%) are independent of variations due to protein loading and staining, the average of the normalized volume percentage of each spot for n=6 patients in each group and n=6 controls were used to calculate the EF of differentially expressed proteins. The NS:S ratio was calculated to demonstrate the fold-change in protein expression in NS compared with S from onset (day of diagnosis) until recovery/mortality, and early stages of sepsis 24 h-day 3 (Table IV). Haptoglobin (Hp; spot 229, $P<0.012$; α1 antitrypsin (A1AT), orosomucoid 1/ α1 acid glycoprotein (ORM1) and S100A9 ($P<0.004$), and serum amyloid A (SAA) exhibited a ≥1.5 fold increase, whereas transthyretin (TTR) (spot 237, $P<0.03$; and Hp (spot 401, $P<0.005$) exhibited a ≥1 fold decrease, from the early stages until mortality in NS (Fig. 2).

Biological function and protein network analysis. Cellular and biological functions of the six differentially expressed proteins were analyzed, where gene ontology slim analysis data of differentially expressed proteins in sepsis were obtained using mappings from the gene ontology (GO) consortium website (http://geneontology.org), shown in (Fig. 3). These proteins were identified to possess mostly cytoplasmic (23.5%) and extracellular functions (35.2%). Their biological functions predominantly involved the regulation of biological processes (14.6%), response to stimulus (14.6%), defence response (12.1%) and transport (12.1%). The present study sought to identify any possible network interactions between identified proteins using GeneMANIA 3.1.2.6 software (http://www.genemania.org) at the genomic and proteomic level, where GO based weighting was applied to detect maximum connectivity between the input genes, based on their biological process, molecular function and cellular component-based function. All six proteins, S100A9, SAA, Hp, TTR, SERPINA1, and ORM, were shown to be interacting closely in a protein interaction network, indicating co-expression of 91.34% and co-localization of 8.66%, where expression was calculated as the Pearson correlation coefficient (Fig. 4).

Analysis of mRNA expression levels. RT-qPCR analysis of the mRNA isolated from whole blood of six patients revealed a ≥2 fold increase in mRNA expression levels (NS:S fold change) of acute phase proteins, S100A9 (2.13), TTR (2.86), SAA (1.84), A1AT (1.4), ORM1 (1.68; $P\leq0.05$) and inflammatory markers, interleukin (IL)-6 (2.5), IL-10 (1.70), prepronociceptin (PPN; 1.6; all $P\leq0.0001$ during early stages). By contrast, Hp (0.59) and Toll-like receptor 4 TLR4 (0.30) exhibited decreased levels during early stages in NS (Figs. 5 and 6). Hp (protein, 0.59; mRNA, 0.62; $P\leq0.05$) and TTR (protein, 3.9; mRNA, 2.86; $P\leq0.05$) showed a correlation between protein and mRNA expression levels during the early stages, whereas the other genes exhibited no significant correlation.

Discussion

An emphasis on the identification of differentially expressed proteins during the early stages of sepsis is useful in identification of marker(s) for predicting potential NS. The ratio of male patients admitted with bacterial sepsis dominated female patients during the present study; hence, the present study

Table III. MALDI-TOF analysis of proteins differentially expressed in sepsis survivors and non-survivors.

Spot ID	Protein identified	pI	Mol. Wt. (kDa)	Mascot score	Accession number and ID[a]	Attributes (half-life)
F3	α1 acid glycoprotein	4.9	49	65	P02763	Acute phase protein, elevated in inflammation (5 days)
237	Transthyretin (prealbumin)	5.5	15	66	P02766, TTHY_HUMAN	Negative acute phase protein (12-24 h)
246	S100A9	5.7	13	70	P06702, S10A9_HUMAN	Prominent role in the regulation of inflammatory processes and immune response (5 h)
287	Hemoglobin subunit β	7.6	14	59	P68871, HBB_HUMAN	Coagulation and complement pathway
401	Haptoglobin	5.8	45	63	P00738, HPT_HUMAN	Acute phase protein (3.5-5 days unbound; 30 min bound)
228	Haptoglobin	5.2	23	67		
229	Haptoglobin	5.3	23	56		
230	Haptoglobin	5.9	23	82		
231	Haptoglobin	6.0	23	62		
Spot I	α 1 antitrypsin/ SERPINA1	5.3	46	96	P01009, A1AT_HUMAN	Acute phase protein (4.5 days)
M	α1 antitrypsin	3.5	46	91		
252	Serum amyloid A	6.3	11	79	P0DJI8 SAA1_HUMAN	Positive acute phase protein (90 min)

[a]Obtained from SWISS-PROT. MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; Mol. Wt., molecular weight; ID, identification; pI, isoelectric point.

Table IV. Expression factors of differentially expressed proteins identified in sepsis survivors and non-survivors from onset until recovery or fatality.

Spot ID	Protein identified (early stages)	NS:S ratio (onset until recovery or death)	NS:S ratio	P-value
401	Haptoglobin	0.62	0.53	0.005[a]
229	Haptoglobin	0.39	0.51	0.012[b]
I	α 1 antitrypsin/SERPINA1	0.92	1.03	0.87
F3	Orosomucoid 1	0.69	1.05	0.72
246	S100A9	1.36	1.68	0.004[a]
237	Transthyretin	3.96	2.03	0.03[b]
244	Transthyretin	1.05	1.48	0.01[b]
251	Serum amyloid A	0.69	0.99	0.76

Expression factors were calculated as the ratio of the mean of the normalized spot volume (%) divided by the mean control spot volume (%). The ratio of the normalized mean ± standard error of the mean values of the non-survivor, vs. survivor was calculated to show the fold change in protein expression in non-survivors compared with the survivors from onset (day of diagnosis) to recovery and the early stages of sepsis. ([a]P<0.01; [b]P<0.05).

was focused on an assessment of the serum protein profiles of male patients with severe bacterial sepsis. Currently, CRP and PCT are the only approved biomarkers used to monitor the progression of sepsis. PCT has a better profile as an early diagnostic marker for detection of bacteremia. Effectiveness of PCT in differentiating between Gram-positive and Gram-negative

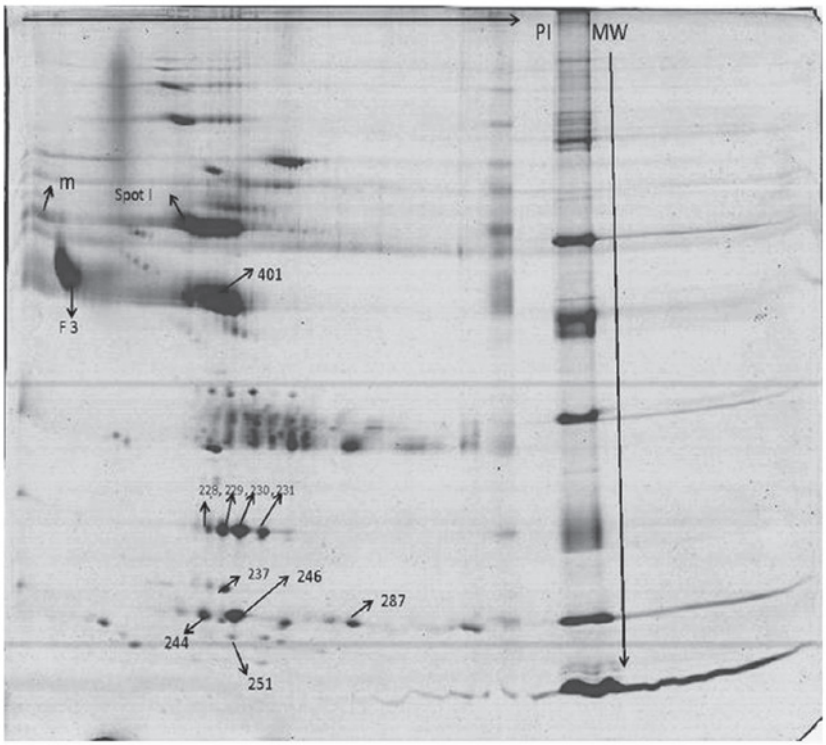

Figure 1. A representative 2D gel image of albumin-depleted serum proteins from patients with sepsis. Albumin-depleted serum samples from patients with sepsis were separated by 2D electrophoresis. The proteins, resolved by isoelectric focusing using 11 cm, 3-10 pH range immobilized pH gradient strips, were separated by gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10-14%). The gels were stained with Coomassie Brilliant Blue (G250) and analyzed by Image master 2D Platinum. The spots revealed to have differential spot volumes, as highlighted in the image, in survivors compared with the non-survivors were identified by matrix-assisted laser desorption ionization-time of flight. Spot I, α1 antitrypsin; 401/228/229/230/231, haptoglobin; F3, α1 acid glycoprotein 1; 237/244, transthyretin; 246, S100A9; 287, hemoglobin subunit β; 251, serum amyloid A.

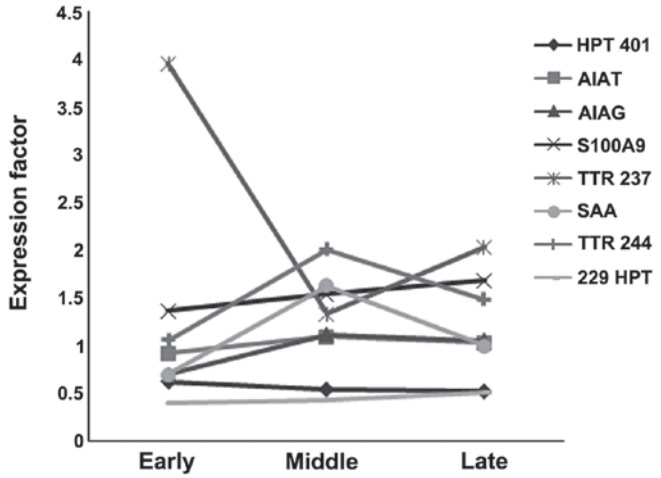

Figure 2. Graph representing changes in the levels of acute phase proteins in early, middle and late stages of sepsis in non-survivors. The ratio of the normalized mean ± standard error of the mean values of non-survivor against survivor was calculated to show the fold change in protein levels in non-survivors compared with survivors during the early (days 1-3), middle (days 3-5) and late stages (final change in protein levels) of sepsis. HPT, haptoglobin; AIAT, α 1 antitrypsin; AIAG, α1 acid glycoprotein; TTR, transthyretin, SAA, serum amyloid A.

sepsis remains unclear (20-23). Previous studies suggested that, although PCT values showed a significant difference between S and NS (24), only APACHE II and male gender were shown to be independent predictors of mortality due to sepsis (25). Also, in the present study, it was observed that the PCT values revealed almost no variation in S and NS, even in the early stages (Table I). The main objective of the present study was to identify an early marker to diagnose susceptibility of a sepsis patient to mortality.

To pursue this objective, the present study first separated daily serum samples from sepsis patients by two-dimensional gel electrophoresis and compared protein profiles of S and NS. A total of 12 differentially expressed proteins were identified from the sera of sepsis patients taken from the onset until recovery/death, where Hp was observed in 5/12 spots, A1AT in 3/12 spots and TTR in 2/12 spots. The six differentially expressed proteins identified in the present study were grouped based on their family and function, as follows: i) α1 globulins, including A1AT, ORM1, SAA; ii) α2 globulins, including pre-albumin, TTR, Hp; iii) Danger-associated molecular patterns/Alarmins-Calgranulin/S100A9.

The majority of the differentially expressed proteins identified in the present study are components of inflammatory processes and the immune response, as demonstrated in protein centre analysis data utilizing the GO database (http://www.geneontology.org) and Kyoto encyclopedia of Genes and Genomes pathway (http://www.genome.jp/kegg/pathway.html). All the six proteins are known to be involved in the host defence response and regulation of inflammatory processes (Fig. 3) (26,27). Further analysis using the GeneMANIA 3.1.2.6 software database (http://www.genemania.org) revealed close interactions between S100A9, SAA, Hp, TTR, SERPINA1 and ORM1 at the genomic

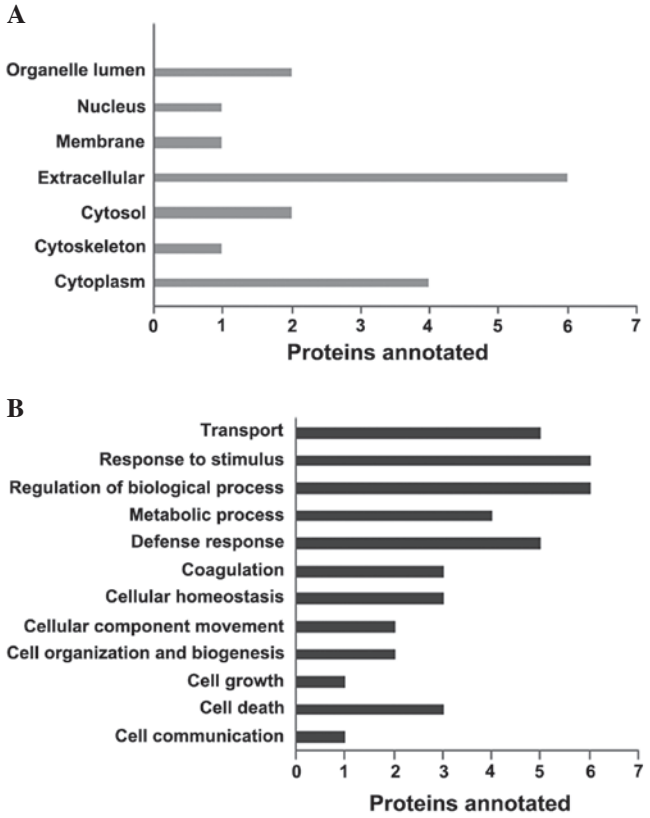

Figure 3. Gene ontology analysis of the differentially expressed proteins identified in patients with sepsis. A bar chart is shown representing (A) the cellular and (B) biological processes of serum amyloid A, transthyretin, haptoglobin, α-1 acid glycoprotein, α-1 antitrypsin and S100A9, identified to be differentially expressed proteins between survivors and non-survivors, as analyzed by protein centre software version 3.10.

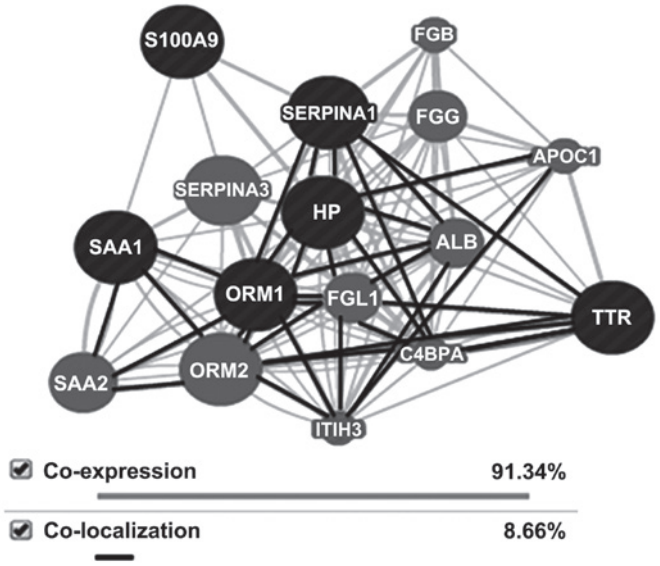

Figure 4. Network interaction between identified proteins as analyzed by GeneMANIA 3.1.2.6 software. Any possible network interaction between the differentially expressed proteins, serum amyloid A, transthyretin, haptoglobin, α-1 acid glycoprotein, α-1 antitrypsin and S100A9, in sepsis at the genomic and proteomic level was assessed. Grey = co-expression; dark = co-localization. AIAT, α 1 antitrypsin; ALB, albumin; APOC1, apolipoprotein C1; C4BPA, complement component 4 binding protein; FGB, fibrinogen β chain; FGG, fibrinogen γ chain; FGL1, fibrinogen-like 1; HP, haptoglobin; ITIH3, inter-α (glubulin) inhibitor H3; ORM, orosomucoid 1; SAA, serum amyloid A; SERPINA, α1 acid glycoprotein; TTR, transthyretin.

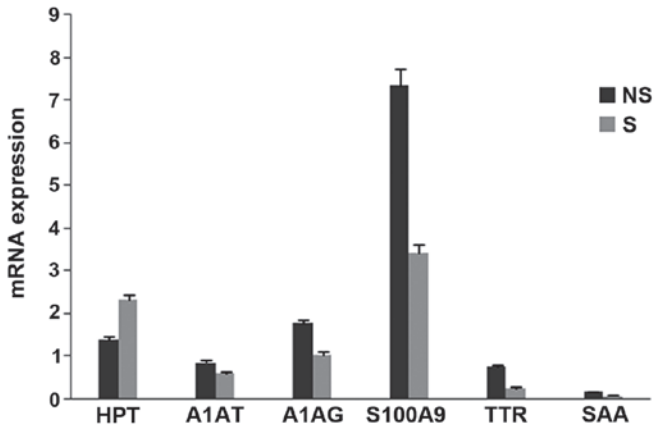

Figure 5. Graph representing changes in whole blood mRNA levels of acute phase proteins in early stages of sepsis in NS and S. Whole blood mRNA expression levels for six differentially expressed proteins were determined in the blood samples of S and NS by reverse transcription-quantitative polymerase chain reaction. The data are represented as mean of the $2^{-\Delta\Delta Cq}$ values and the mRNA levels are expressed as the NS:S ratio (fold change) of acute phase proteins, S100A9 (2.13), TTR (2.86), SAA (1.84), A1AT (1.4), ORM1 (1.68). ($P \leq 0.05$, vs. control blood from a healthy donor). NS, non-survivors; S, survivors; HPT, haptoglobin; AIAT, α 1 antitrypsin; AIAG, α1 acid glycoprotein; TTR, transthyretin, SAA, serum amyloid A.

and proteomic level (Fig. 4). Delayed increase of proteins, including S100A9, SERPINA1, TTR, SAA and Hp, during the early stages of sepsis in NS indicated their potential role in sepsis survival (Fig. 2). Drug metabolism and drug delivery are critical in sepsis treatment. The present study demonstrated that decreased expression of drug binding proteins, including α1-acid glycoprotein, is correlated with the severity of sepsis.

Acute phase response leads to an increase in serum globular proteins, which are grouped into α1 globulins, α2 globulins, β-globulins secreted by the liver, and γ globulins. The pattern of expression of α1 and α2 globulins in the present study indicated their possible role in the early stages of sepsis in male patients (Fig. 2). SAA is a precursor for amyloid A, generally shown to have an immunomodulatory effect and to be important for binding to Gram-negative bacteria, thereby facilitating their uptake by macrophages and neutrophils (28,29). In the present study, low SAA levels were observed in more NS during early sepsis compared with S. The general trend was observed as a rise during the middle stages and gradual decrease towards recovery or death in sepsis. Similar results have been reported previously, where SAA levels rise within 24 h following infection and then tend to decrease slowly (30). However, the present study observed that SAA levels remained slightly higher in NS, even during the late stages of sepsis, when compared with S. Although certain studies suggest that SAA is a more sensitive marker for inflammatory disease (31), further investigations on its role in larger cohort-based studies would provide more insights in understanding the role of SAA as a potential marker in evaluating the severity of sepsis.

A1AT has anti-proteolytic activity and is known to inhibit particular serine proteases, which increase during inflammation. This protein serves a prominent role in the complement and coagulation pathways. The present study observed an

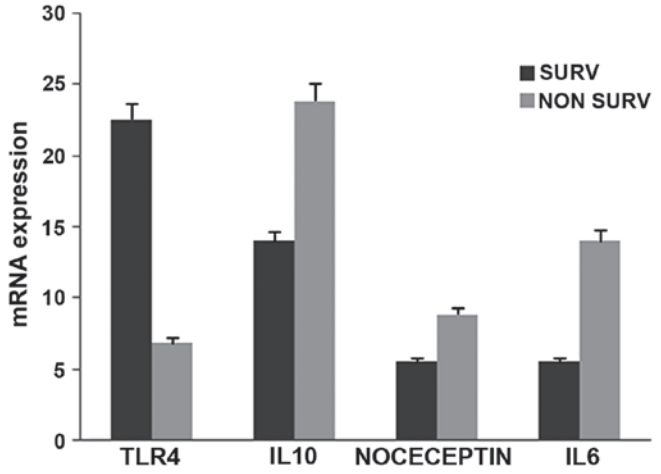

Figure 6. Graph representing changes in whole blood mRNA levels of inflammatory markers in early stages of sepsis in NS and S. Whole blood mRNA expression levels for six differentially expressed proteins were measured in the blood samples of S and NS by reverse transcription-quantitative polymerase chain reaction. The data are represented as mean of the $2^{-\Delta\Delta Cq}$ values and the mRNA levels are expressed as the NS:S ratio (fold change) of the inflammatory markers, TLR4 (0.30), IL-6 (2.5), IL-10 (1.70), NOCECEPTIN (1.6). P<0.0001, vs. control blood from healthy donor. S (SURV), survivor; NS (Non Surv), non-survivor; TLR4, Toll-like receptor 4; HPT, haptoglobin; AIAT, α 1 antitrypsin; AIAG, α1 acid glycoprotein; TTR, transthyretin, SAA, serum amyloid A; NOCICEPTIN, prepronociceptin.

overall rise in A1AT in NS compared with S from the onset until the recovery/mortality. NS exhibited a two-fold reduction in the expression of A1AT levels during the early days of sepsis compared with S. Elevated A1AT values are observed predominantly due to an acute-phase reaction to infection and inflammation, suggesting that increased protein degradation in NS leads to lower levels compared with S.

α1 acid glycoprotein, identified originally as orosomucoid (ORM), is a mucoprotein present in human plasma belonging to the immunoglobin superfamily (32,33). Elevated levels of ORM1 are a characteristic feature of inflammatory responses (34,35). The expression levels of ORM1 are known to be lower in sepsis patients who are unable to recover (36). The present study observed comparatively elevated ORM1 levels in S during early stages, followed by a fall in levels during recovery, unlike NS, where the levels gradually increased towards late stages of sepsis, mostly being stable (prior to mortality). ORM1 may be superior to CRP in terms of investigating the progress of sepsis, since increase in plasma ORM1 levels are associated with an increased mortality rate (36). Comparatively, an increase in ORM1 levels, a basic drug binding protein, in S indicates that drug delivery may play a critical role in sepsis survival during the early stages.

Hp is an acute phase protein exhibiting an α2 glucoprotein structure. Plasma Hp levels are used in the diagnosis of hemolytic events in addition to acute and chronic infections. Although the majority of the previous reports have shown higher levels of serum Hp in neonatal sepsis, due to its low specificity and sensitivity, its application in clinical diagnosis remains under investigation (37). It was revealed that serum Hp levels are elevated in sepsis S during the early stages of sepsis compared with NS.

TTR, also known as pre-albumin, is a thyroxine-binding protein, which also aids in the transport of vitamin A by forming a complex with retinol-binding protein (38). The present study observed elevated levels of TTR in NS during the early stages, followed by its gradual decrease towards mortality. In the present study, TTR levels remained higher from the middle until the end stages of sepsis in S in contrast with NS, where the levels decreased from the middle until the end stages, indicating that elevated levels of TTR may be considered relevant in monitoring sepsis survival.

The pattern recognition receptors of the innate immune response, which recognize endogenous mediators, are released in response to injury, warning the host and are termed 'Alarmins' or danger-associated molecular patterns. S100A9 or myeloid-related protein 14, is an example of an alarmin which amplifies the pro-inflammatory response through TLR4. S100A9 was observed to increase during the early stages of sepsis and remained unchanged during the middle to recovery stages in S, whereas NS exhibited increased levels towards septic shock (39). Although the biological functions of these proteins are not completely understood, they appear to depend on interactions with receptor for advanced glycation end products and TLR4. An increase in S100A9-like alarmins in serum act as indicator of sepsis, and its delayed increase in NS may represent the severity of sepsis.

Gene expression profiling to identify any possible significant correlation between the mRNA and protein expression levels of the six differentially expressed proteins was performed using qPCR, where Hp and TTR exhibited a correlation between protein and mRNA levels in the sepsis patients, while the other four proteins showed no significant correlation. The comparison signifies limited correlation between mRNA and protein expression levels, which may be attributed to the varied half-lives of proteins, the post-translational modifications involved in turning mRNA into proteins (40,41) and, finally, differing experimental conditions. All this may limit the correlation between mRNA and protein expression levels. Several previous studies have demonstrated that little or no correlation is established between mRNA and protein levels (42,43). Protein synthesis, degradation and also protein turnover may vary significantly, depending on a number of different conditions (44). Significant heterogeneity is present, even within similarly functioning proteins (45). For example, the Hp genotype exists in isoforms Hp1-1, Hp 2-1 and Hp 2-2, and their prevalence is observed to vary according to geographic distribution; additionally, Hp1-1, containing more αβ chains, was shown to bind more hemoglobin compared with the other two types (46).

Proinflammatory cytokines, IL1-β, IL6 and TNF, and anti-inflammatory cytokines, IL10, IL-1 receptor antagonists and soluble TNF receptors, serve an important role in mediating sepsis. IL-6 is a cytokine that is released by macrophages, endothelial cells or fibroblasts, and appears to be the most efficient stimulator of the production by the liver of the acute phase proteins in response to IL-1 and/or TNF α. IL10 was reported to be high in NS and a high IL-10:TNF-α ratio was associated with mortality, indicating that the anti-inflammatory cytokine, IL-10, is a predictor for severity and fatal outcome (47,48). Increased IL-10 levels are also known to be associated with a positive outcome in sepsis, and also as a good marker to

study the severity of sepsis, as suggested by studies based on murine models. By contrast, IL-10 knockout mice revealed no difference in the survival rate. In the present study, increased IL-10 and IL-6 expression was observed in NS of sepsis. This revealed no effect on the outcome of the patient.

Nociceptin/orphanin FQ (N/OFQ) is a 17-amino-acid opioid-associated peptide, which is produced from proteolytic cleavage of PPN/orphanin FQ (PPN/OFQ). Activation of nociceptin-NOP signaling is known to induce production of inflammatory mediators, which leads to altered expression of cytokines. Human peripheral blood mononuclear cells and polymorphonuclear leukocytes express mRNA transcripts encoding both PPN/OFQ and the NOP (nociceptin receptor). LPS binds its receptors CD14, TLR4 and myeloid differentiation protein-2 on immune cells, and is known to rapidly induce the mRNA expression of PPN/OFQ (49-51), which is observed to be reversed on inhibiting TLR4. To determine the relevance of TLR4 and PPN/OFQ in bacterial sepsis, in the present study their mRNA expression levels in patient blood samples were screened for in early sepsis. Compared with S, the expression levels of TLR4 were four-fold lower in NS samples, whereas PPN/OFQ levels were 0.38-fold higher in NS, possibly indicating increased conversion of the precursor into N/OFQ. This observation confirms previous findings, where Williams *et al* (52) identified higher nociceptin levels in critically ill patients who underwent gastrointestinal surgery.

Since Asians respond differently to most diseases when compared with other races, a definite understanding of immunological status and pathogenesis of sepsis in these populations is indispensable. The present study provided a brief insight into possible differential expression and correlation among the proteins involved in sepsis in terms of survivability. It is important to have a laboratory method which is rapid, specific and sensitive enough to predict sepsis mortality at an early stage. A biomarker-based algorithm (53) would become predictive if a population-specific genetic marker, which predisposes the patients to sepsis, is identified.

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