Proteomics Reveals Age-related Differences in the Host Immune Response to Sepsis

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Abstract

Sepsis is commonly caused by community-acquired pneumonia (CAP) and may develop into severe sepsis, characterized by multiple organ failure. The risk of severe sepsis among CAP patients and subsequent mortality increases sharply after the age of 65. The molecular mechanisms associated with this age-related risk are not fully understood. To better understand factors involved with increased incidence and mortality of severe sepsis in the elderly, we used a nested case-control study of patients enrolled in a multicenter observational cohort of 2,320 participants with CAP. We identified a total of 39 CAP patients 50-65 and 70-85 years old who did or did not develop severe sepsis. Plasma samples were obtained on presentation to the emergency department and prior to therapeutic interventions. A semi-quantitative plasma proteomics workflow was applied which incorporated tandem immunoaffinity depletion, iTRAQ labeling, strong cation exchange fractionation, and nanoflow-liquid chromatography coupled to high resolution mass spectrometry. In total, 772 proteins were identified, of which, 58 proteins exhibit statistically significant differences in expression levels amongst patients with severe sepsis as a function of age. Differentially-expressed proteins are involved in pathways such as acute phase response, coagulation signaling, atherosclerosis signaling, lipid metabolism, and production of nitric oxide and reactive oxygen species. This study provides insight into factors that may explain age-related differences in incidence of severe sepsis in the elderly.

Keywords

Introduction

Sepsis is a systematic inflammatory state triggered by infection. The additional diagnosis of multiple organ failure and hypoperfusion (e.g., hypotension, decreased urine output) along with sepsis results in severe sepsis[1]. One of the leading causes of sepsis is community-acquired pneumonia (CAP)[2-4]. The presence and severity of organ failure is one of the most important determinants of mortality following sepsis[5-10]. Severe sepsis affects ~750,000

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persons annually in the United States[6-11] and is one of the most common causes of death in intensive care units[6-10].

Kellum and coworkers have shown that the incidence of severe sepsis and mortality rate increases sharply after the age of 65[12, 13]. For example, CAP patients 85 years old have a 2.8fold increased risk of severe sepsis and a 17-fold increased mortality rate compared to patients 50 years old[2]. The notable age-related differences in severe sepsis risk may be explained in part by immunosenescence underlying chronic disease. Immunosenescence is manifested in the elderly through decreased numbers of T cells, impaired B-cell function, increased apoptosis of neutrophils, and reduced bactericidal response of macrophages[13-15]. Most studies that examine age-related differences in immune response using animal models and humans have largely focused on either the adaptive immune response or select pathways (inflammatory, coagulation, and fibrinolysis markers) in the innate immune response[14-20]. A comprehensive assessment of differences in the immune response has not been conducted. Trials testing immunomodulating therapies for sepsis in broad populations have failed to consistently improve outcomes of sepsis patients[21]. An alternative approach is to personalize sepsis therapies based on host characteristics, such as age. To further the development of such a personalized approach, a better understanding of the differences in immune response due to age is necessary.

Herein, we conducted a nested case-control study using patients enrolled in an observational cohort of CAP. A semi-quantitative plasma proteomics workflow previously developed in our laboratory[22], which included tandem immunoaffinity depletion, isobaric tags for relative and absolute quantitation (iTRAQ) labeling, strong cation exchange (SCX) fractionation, and nanoflow liquid chromatography (LC) coupled to high resolution mass spectrometry (MS), was applied to patient samples. We analyzed plasma proteins on presentation to the emergency department to compare the acute immune response. First, proteins were identified that differed between those who did or did not develop severe sepsis (within 90 days of hospitalization) among younger and older adults separately. We hypothesized that there may be differentially-expressed proteins that are unique to the younger or older adults which would help to explain the increased risk of severe sepsis in older adults. In addition to identifying these sets of proteins, differentially-expressed proteins that are common to the younger and older adult groups were also identified. Interestingly, the direction of fold-change observed for these common proteins varies depending on patient age and thus may explain higher risk of severe sepsis in older adults. These results and the implications of this study are presented herein.

Experimental procedures

Ethics statement

The Institutional Review Boards at the following hospitals approved the study: Pennsylvania: Allegheny General Hospital, Jefferson Hospital/SHHS, Mercy Hospital, St. Clair Memorial Hospital, St. Francis Medical Center, Sewickley Valley Hospital, University of Pittsburgh Medical Center (UPMC) Braddock, UPMC Horizon, UPMC Lee, UPMC McKeesport, UPMC Passavant, UPMC Presbyterian, UPMC Shadyside, UPMC Southside, UPMC St. Margaret, West Penn Hospital; Connecticut: Bridgeport Hospital, Hartford Hospital, Milford Hospital, New Britain General Hospital, Norwalk Hospital, Yale-New Haven Hospital; Tennessee: Methodist Health Care (single IRB approval for three Methodist University sites); Michigan: Henry Ford Health System, Detroit Receiving/Sinai-Grace, Wayne State. Written, informed consent was obtained from all participants or by proxy.

Study design and patients

We conducted a nested case-control study using patients enrolled in the GenIMS study[23]. GenIMS is a prospective multicenter observational cohort of patients with CAP enrolled in emergency departments (EDs) of 28 academic and community hospitals in four US regions, including southwestern Pennsylvania, Connecticut, southern Michigan and western Tennessee. Details of this study including eligibility criteria have been described previously[2, 23].

To compare differences in immune response across different age groups and between patients with and without severe sepsis, we identified a total of 39 patients from four groups. These included: 1) patients 50-65 years old who did not develop severe (hereafter referred to as young controls [YC]), 2) patients 70-85 years old who did not develop severe sepsis (hereafter referred to as old controls [OC]), 3) patients 50-65 years old who developed severe sepsis within 90 days after presentation to the ED (hereafter referred to as young severe sepsis [YS]), and 4) patients 70-85 years old who developed severe sepsis within 90 days after presentation to the ED (hereafter referred to as old severe sepsis [OS]). To ensure that differences in immune response are attributed to age and not to ethnic differences or underlying chronic diseases, only whites and matched patients according to chronic disease burden were included (Table S1). Approval for the participation of human patients was obtained by the Institutional Review Board of the University of Pittsburgh and other participating sites.

Plasma samples and tandem MARS depletion

Plasma samples were obtained on Day 1 when the CAP patients were admitted to the ED and prior to most interventions to ensure that differences in immune response are not affected by therapeutic strategies. Negative results were obtained in the blood culture test used to identify the pathogenic source of the samples obtained from CAP patients. The Hu 6 MARS column (Agilent; Santa Clara, CA) depletes serum albumin, IgG, α1-antitrypsin, IgA, transferrin, and haptoglobin proteins. An injection amount of 60 μL of crude plasma was applied to the MARS column and after the initial depletion flow-through fractions were concentrated with a 5K molecular weight cutoff concentrator (Agilent) at 4695 g at 4°C for 1.5 hours. Samples were then stored at -80 °C or re-injected onto the MARS column for tandem MARS depletion. The second flow-through fractions (hereafter referred to as TMD) were concentrated and protein concentrations were measured using the BCA protein assay.

Protein digestion

Protein was denatured with an extraction buffer (0.2 M Tris, 8 M urea, 10 mM $CaCl_2$, pH 8.0), reduced with 1:40 molar excess of dithiothreitol (DTT) for 2 h at 37 °C, and then alkylated with 1:80 molar excess of iodoacetamide (IAM) for 2 h on ice. The alkylation reaction was quenched by adding 1:40 molar excess of Cysteine and the mixture was incubated at room temperature for 30 min. Tris buffer (0.2 M Tris, 10 mM $CaCl_2$, pH 8.0) was added to dilute the urea concentration to 2 M. Each sample was incubated with bovine TPCK-heated trypsin at 1:50 substrate: enzyme ratio for 24 h at 37 °C.

iTRAQ labeling

Digested samples were desalted with an HLB cartridge (Waters; Milford, MA) and dried by centrifugal evaporation. Each sample was labeled with an iTRAQ reagent following the manufacturer's protocol (Applied Biosystems; Foster City, CA) with slight modifications. Briefly, each iTRAQ reagent was solubilized with 70 μL ethanol and transferred to peptide mixtures. After 1.5 h of incubation, the reaction was quenched with water. Labeled samples

were mixed in 1:1:1:1 ratios for iTRAQ reagents that generate reporter ions at *m/z* 114:115:116:117, respectively.

SCX fractionation

SCX fractionation was carried out on a PolySulfoethyl A 100 mm × 2.1 mm, 5 μm, 200 Å column (The Nest Group, Inc.; Southborough, MA) with buffers as follows: mobile phase A was 5 mM monopotassium phosphate (25% v/v acetonitrile, pH 3.0), and mobile phase B was 5 mM phosphate, 350 mM potassium chloride, (25% v/v acetonitrile, pH 3.0). Dried iTRAQ labeled samples were resuspended in 300 μL of mobile phase A and injected onto the SCX column. The gradient for SCX was: 0-3 min, 0% B; 3-45 min, 0-75% B; 45-50 min, 75-100% B; 50-55 min, 100% mobile phase B; 55-56 min, 100-0% B; 56-106 min, 0% B. Thirteen SCX fractions were collected and each fraction was desalted with an HLB cartridge (Waters).

LC-MS/MS analysis

Online desalting and reversed phase chromatography was performed with a Nano2D-LC system equipped with an autosampler (Eksigent; Dublin, CA). Mobile phase A and B for these analyses were 3% (v/v) acetonitrile with 0.1% formic acid and 100% (v/v) acetonitrile with 0.1% formic acid, respectively. SCX fractions (5 μL)were loaded onto a trapping column (100 μm i.d. × 2 cm), which was packed in-house with C_{18} 200 Å 3μm stationary phase material (Michrom Bioresource Inc.; Auburn, CA) at 3 μL.min^{-1} in 3% mobile phase B for 3 min. After desalting, the sample was loaded onto an analytical column (75 μm i.d. × 13.2 cm), which was packed in-house with C_{18} 100 Å 3μm stationary phase material (Michrom Bioresource Inc.). The gradient was as follows: 0-5 min, 10% mobile phase B; 5-75 min, 10-30% B; 75-95 min, 30-60% B; 95-100 min, 60-90% B; 100-105 min, 90-10% B; 110-120 min, 10% B. The LC eluent was analyzed with positive ion nanoflow electrospray using a LTQ-Orbitrap Velos mass spectrometer (Thermo-Fisher Scientific, Waltham, MA). Data-dependent acquisition parameters were as follows: the MS survey scan in the Orbitrap was 60,000 resolution over 300-1800 *m/z*; the top six most intense peaks in the MS survey scan were isolated and fragmented with CID and HCD; CID was performed in the ion trap with normalized collision energy 35%; HCD was recorded in the Orbitrap with normalized collision energy 45% and 7,500 resolution; dynamic exclusion was enabled and a repeat count of 2 for a duration of 60 seconds was allowed and selected ions were placed on an exclusion list for 61seconds. Each SCX fraction was subject to triplicate LC-MS/MS analysis.

Data analysis

RAW files were analyzed with Proteome Discoverer 1.2 software (Thermo). Both CID and HCD spectra were used to obtain sequence information against the Uniprot human database (04/25/2010, 20295 sequences). Sequest search parameters were as follows: two maximum trypsin miscleavages; precursor mass tolerance 10 ppm; fragment mass tolerance 0.8 Da; static modifications were iTRAQ-4plex/+144.102 Da (N-terminus, Lys), and carbamidomethyl modification/+57.021 Da (Cys); dynamic modification of iTRAQ-4plex/ +144.102 Da (Tyr). Decoy database searching was employed to generate medium (p<0.05) and high (p<0.01) confidence peptide lists. All the peptides with medium and high confidence were used to identify and quantify proteins. The reporter ions (i.e., m/z 114-117) were identified with the following parameters: centroid with smallest delta mass, 20 ppm for reporter ion mass tolerance. The isotope correction was employed according to the manufacturer's protocol (AB Sciex). Additionally, protein ratios in each experiment were normalized based on the protein median ratio option in the software (i.e., individual protein value is normalized against the median ratio value obtained across all proteins identified in a

given experiment). Only proteins with at least two spectral counts in a technical replicate were considered for further analysis.

Statistics

Coefficient of variation (CV) values were calculated for reporter ion ratios (e.g., 115/114, 116/114, and 117/114) of proteins quantified in at least six iTRAQ experiments. The mean CV value across the iTRAQ experiments was calculated and used as the total biological variation, S_b, which was 0.60 in this study. The technical variation, S_t, was calculated for proteins quantified in at least two LC-MS/MS analyses within an individual iTRAQ experiment, which was 0.11 in this study. The relation between the fold change (F), random variation (S), biological variation (S_b), and technical variation S_t is expressed by the formula[24]

$$n=2\frac{(Z+T)^2 S^2}{(F-1)} \quad (1)$$

$$S^2=\frac{S_b^2}{n}+\frac{S_t^2}{nm} \quad (2)$$

The quantities Z and T depend on the power of the test and the significance level, respectively. When the power of the test and significance level are set as 0.8 and 0.05, respectively, the value of $(Z+T)^2$ will be $\sim 10^{24}$. n and m (i.e., m is set as 3 in these studies) are the number of biological and technical replicates, respectively. Such that formula (1) approximates to[24]

$$10=20\frac{S^2}{(F-1)^2} \quad (3)$$

Solve formulas (2) and (3),

$$F=(2\frac{3S_b^2+S_t^2}{3n})^{1/2}+1 \quad (4)$$

From this power analysis, fold-change cutoff was calculated based on the number of biological replicates (i.e., n) in which the proteins were quantified. Stringent filter criteria were applied to generate a list of statistically significant differentially-expressed proteins as follows: 1) proteins identified and quantified in at least six biological replicates, 2) CV values 0.60, and 3) fold-change cutoff dependent upon n as such a) 1.27 or 0.79 (n=10), b) 1.28 or 0.78 (n=9), c) 1.30 or 0.77 (n=8), d) 1.32 or 0.76 (n=9), e) 1.35 or 0.74 (n=6).

Western blotting analyses

The changes in the expression of C-reactive protein (CRP), apolipoprotein CIII (ApoCIII), and fibrinogen alpha chain (FAC) were subject to Western blotting analysis. Twenty μg of TMD proteins was denatured in an appropriate sample buffer and electrophoretically separated on a Criterion precast gel (Biorad Laboratories; Hercules, CA) at 140 V. Proteins from the gel were transferred onto a nitrocellulose membrane paper using a Fast-Transfer Blot System (Biorad). Blots were washed three times in Wash blot. BSA blocking solution

(3%) was added to the membrane and incubated on a rocker for 2 h. A 1:5000 dilution of mouse monoclonal anti-CRP primary antibody (Sigma Aldrich; St. Louis, MO), 1:5000 dilution of rabbit polyclonal anti-Apo CIII primary antibody (Abcam; Cambridge, MA), or 1:2500 dilution of rabbit monoclonal anti-FAC primary antibody (Abcam) was added and incubated at 4 °C overnight. The blot was rinsed and incubated with a 1:7500 dilution of anti-mouse or anti-rabbit IgG alkaline phosphatase secondary antibody (Sigma Aldrich) for 1 h on a rocker. The blot was rinsed and colorometrically developed using 0.51mM 5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt (BCIP) and 0.24 mM nitrotetrazolium blue (NBT). The dried blot was scanned using a Canon scanner, saved as a .TIFF file, and densitometry analyses carried out with Scion Image Software. Within each experiment, the intensity for the sample from each group was normalized to the total blot intensity and used to generate mean and standard deviation values.

Ingenuity pathway analysis

Differentially-expressed proteins were analyzed using Ingenuity Pathway Analysis (IPA, www.ingenuity.com) to generate a list of pathways that are statistically relevant (p< 0.05).

Results

Data characterization and statistical analysis

An iTRAQ-based semi-quantitative proteomics workflow[22] was employed to identify differentially-expressed proteins in 50-65 and 70-85 year old CAP patients who developed severe sepsis compared to those patients that did not (Table S1). The workflow employs tandem MARS depletion on a MARS Hu-6 column to effectively remove high abundance proteins and has been demonstrated to allow deeper probing into the plasma proteome[22]. Patient samples from the four groups (i.e., YC, YS, OC and OS) were randomly assigned to iTRAQ reagents in a blind fashion across ten SCX-LC-MS/MS experiments (Table 1).

Figure 1a shows an example of LC chromatograms for 13 SCX fractions of pooled iTRAQ 4-plex samples. The triplicate LC-MS/MS runs for each fraction are reproducible. Figures 1b-g show example spectra for peaks isolated and fragmented. Doubly-charged peptides at *m/z* 748.9046 (Figure 1b) and 742.9010 (Figure 1c) in SCX fractions seven and six were eluted from the column at t_r57.20 min and 37.26 min, respectively. The CID MS/MS spectra (Figures 1d and e) display a consecutive series of *b*- and *y*-fragment ions used to assign the peptides as [YYTYLIMNK+2H]$^{2+}$ of protein Complement C3 and [GWVTDGFSSLK +2H]$^{2+}$of protein ApoCIII, respectively. A mass increase of 145 Da was observed for b_1 and $y_1$ions for each of the two peptides, indicating iTRAQ labeling at the N-terminus and the presence of lysine at the C-terminus. HCD MS/MS spectra (Figures 1f and g) also contain a series of *b*- and *y*- ions used to confirm the sequence of peptides. The lower *m/z* region of the HCD MS/MS spectra (inserts in Figure 1f and g) shows the intensity of the reporter ions which is used to quantify the peptides. As shown in Figures 1f and g, the ratios across the four groups are 1.0:0.9:1.0:1.0 and 1.0:1.9:2.3:1.4 for reporter ions 114:115:116:117 from Complement C3 and ApoCIII, respectively.

Figure 2 shows a bar graph of the number of proteins and spectral counts (SCs) identified in each of ten biological replicate experiments. Also, the cumulative number of proteins identified in all of the ten experiments is also shown in Figure 2. An average of 283±14 proteins and 59645±4129 spectral counts were identified and similar results were obtained across individual experiments. The total number of proteins identified increases with each pooled sample experiment such that a total of 772 unique proteins were identified from all plasma samples. With more stringent criteria for protein filtering (i.e., at least two unique peptides), a total of 509 proteins were identified. A list of all identified proteins and peptides

is provided in Supplementary Tables S2 and S3, respectively. Based on SCs, the three most abundant proteins are Complement C3 (49,749 SCs), α-2-macroglobulin (47,273 SCs) and ApoAI (28,261 SCs).

Western analysis verification

Western blotting analysis was employed to generate a secondary measurement for several differentially expressed proteins. Proteins involved in the acute phase response (i.e., CRP), coagulation pathway (i.e., FAC) pathway and lipid metabolism (i.e., ApoCIII) were selected. Figures 3a-c display Western blot data for CRP, FAC, ApoCIII, respectively. The histogram plots represent the normalized intensities corresponding to the density of the band spots for each group. The relative abundances (YS:YC:OS:OC) obtained from the Western blotting analysis of CRP, FAC and ApoCIII were 1.0:0.4:0.4:1.8, 1:0.3:0.1:0.5, and 1.0:1.9:2.5:1.2, respectively. These observed Western data were similar to the measured iTRAQ results for these proteins (Table 2). However, suppression of iTRAQ protein ratios in MS/MS experiments is a noted limitation of this quantitative approach[25]. Proteomics workflows to handle suppression issues at the MS^3 level have been recently reported[26-30].

Differentially-expressed proteins

Proteins ratios were obtained from the two age groups: YS/YC in 50-65 years old group and OS/OC in 70-85 years old group using YC and OC as reference channels, respectively. Fifty-eight differentially-expressed proteins (Table 2) were identified in comparisons from both age groups (i.e., YS/YC and OS/OC). Proteins differentially expressed between younger and older adults within each disease group (i.e., OC/YC and OS/YS) are provided in Supplemental Table S5 for interested readers. The measured fold-change values reported in the table include the mean and SD for each protein based on the ratios averaged across all biological replicates. As shown in the Venn diagram (Figure 4), 28 proteins are differentially-expressed in the population of 50-65 year olds (i.e., YS/YC) in which 14 have higher levels and 14 have lower levels in patients with severe sepsis compared to those with CAP. In the population of 70-85 year olds (i.e., OS/OC), 23 proteins are differentially-expressed, in which 16 and seven have higher and lower levels in patients with severe sepsis, respectively. Of the 58 total differentially-expressed proteins, eight are in common amongst both age groups. Interestingly, however the direction of the fold change differs in younger and older adults. Specifically, α-1-antichymotrypsin (A1ACT), ApoE, fibrinogen α chain, C-reactive protein (CRP), and LPS binding protein (LBP) levels were higher in younger adults with severe sepsis but lower in older adults with severe sepsis relative to age-matched controls, suggesting that lower levels of these proteins is associated with increased risk and incidence of severe sepsis in older adults. On the other hand, Apo AII, Apo CIII, and N-acetylmuranoyl-L-alanineamidase have lower and higher levels in younger and older with severe sepsis, respectively, suggesting that higher levels of these proteins may be factors for increased severe sepsis risk in older adults.

Pathway analysis

Using IPA analysis, 19 biological pathways (Figure 5) are significantly over-represented (p<0.05) for the 58 differentially-expressed proteins identified in these studies. The most represented pathways include LXR/RXR activation and acute phase response signaling where by 18 proteins are associated with each of these pathways. Next many differentially-expressed proteins are involved in atherosclerosis signaling, interleukin (IL)-12 signaling and production of NO and reactive oxygen species in macrophages, and endocytosis signaling. Fewer proteins are involved in the remaining biological pathways shown in Figure 5 such as actin cytoskeleton and IL-6 signaling. A list of the proteins associated with specific pathways is provided in Supplemental Table S4.

Discussion

This work investigates the effects of aging on the risk of severe sepsis in the acute plasma proteome of elderly CAP patients. While sepsis can occur as early as the neonatal stage[31, 32], incidence and morbidity increases with age and rises sharply after 65 years[13, 33, 34] presumably due to immunosenescence[14, 34, 35] and high levels of inflammatory proteins[17-20, 36]. However, no significant differences with age have been reported for inflammatory and coagulation proteins and cell surface markers in patients with severe sepsis[2].

Our plasma proteomics study identified 58 differentially-expressed proteins in CAP patients that subsequently developed severe sepsis relative to those who did not in groups of elderly adults (Table 2). We note that some of the differences observed may be due to both a mixture of pre-existing conditions and differences in the initial acute phase response, as our study groups did not include a nonseptic subset. Below is a discussion of the proteins involved the biological pathways such as acute phase signaling, coagulation pathway, and lipid metabolism. Other pathways are briefly discussed and the implications of these changes for understanding aging and severe sepsis in the elderly are presented.

Acute phase response

Sepsis is a pro-inflammatory state which is characterized by elevated levels of pro-inflammatory cytokines, such as IL-6, IL-10 and tumor necrosis factor (TNF)-α[11]. These pro-inflammatory cytokines regulate acute phase responses and the expression of acute phase proteins (APPs)[37]. Altered expression of APPs has been demonstrated in sepsis[16, 38-42]. For example, increased levels of α-1-acid glycoprotein (A1AG), A1ACT, and LBP and decreased levels of transthyretin (TTR) are observed in 65 year old sepsis patients[39, 40] and cecal ligand and puncture (CLP) mouse[41] and pig models[42]. It also appears that acute phase response increases with the disease severity. For example, sepsis patients have higher levels of CRP and lower levels of serum amyloid A4 relative to patients with systemic inflammatory response syndrome (SIRS), another precursor to sepsis[16, 38].

In our studies, 18 differentially-expressed proteins including CRP, LBP, A1ACT, and TTR are involved in acute phase response (Figure 5 and Table S4) and the levels in younger adults are consistent with other studies[16, 38-42]. For example, CRP (fold-change YS/YC 3.268±1.070), LBP (2.201±0.502), A1ACT (1.577±0.867), and A1AG (2.033±0.426) have higher concentrations while TTR (0.440±0.094) has lower concentrations in younger adults who developed severe sepsis compared to those did not. Also evidenced in our results is that acute phase response also varies depending on patient age. The proteins CRP (0.521±0.221), LBP (0.762±0.321), and A1ACT (0.683±0.183) have lower concentrations in older adults who later developed severe sepsis suggesting that lower expression of these proteins at older age leads to severe sepsis. CRP and LBP have protective effects by neutralizing the toxicity of pathogens[39, 43-46], such that decreased expression of these proteins in older patients may help explain higher risk and mortality of severe sepsis. Both aging and severe sepsis alter the immune system and response to infection[47]. Taken together, these results of acute phase response imply that a hypoinflammatory response in older adults may increase risk of severe sepsis, consistent with other literature reports[11, 48, 49].

Coagulation pathway

Pro-inflammatory cytokines can also activate the coagulation pathway. Briefly, the activation of a series of coagulation factors cleave prothrombin to thrombin, which in turn converts soluble fibrinogen into fibrin, upon which cross-linked fibrin forms blood clots[11, 50].In sepsis patients, pro-coagulation overwhelms fibrinolysis (or anti-coagulation)

leading to the presence of more blood clots[11, 50]. This has been demonstrated by decreased levels of anti- coagulant proteins [e.g., antithrombin III (ATIII), and heparin cofactor II] and increased levels of pro-coagulant proteins [e.g., fibrinogen, Von Willebrand factor (VWF)] in sepsis patients[41, 51-53].

In these studies, elevated levels of fibrinogen alpha chain (2.441±0.579), fibrinogen beta chain (2.102±0.579), fibrinogen gamma chain (2.077±0.720) and VWF (2.047±0.340) were found in younger adults who developed severe sepsis. Fibrinogen has higher concentrations and ATIII has lower concentrations in sepsis[41, 51]. Higher levels of VWF are associated with mortality in sepsis[52]. Higher levels of fibrinogen and VWF may indicate the presence of more blood clots in younger patients, consistent with literature reports[53-55], whom developed severe sepsis. This notion is further supported by decreased concentrations of ATIII (0.596±0.064) and heparin cofactor II (0.707±0.255) in these studies. Interestingly, fibrinogen (0.597±0.129) had lower levels in older adults who developed severe sepsis. Our proteomics results support the notion that an acute response of reduced coagulation activity may be a contributing factor to higher incidence and mortality of severe sepsis found in older adults.

Lipid metabolism

Apolipoproteins play important roles in liver X receptor/retinoid X receptor (LXR/RXR) activation and atherosclerosis signaling. For example, Apo B-100 can suppress the activation of LXR/RXR[56]. The activated LXR/RXR complex enhances the process of reverse cholesterol transport and cholesterol efflux, which transfers accumulated cholesterol from the blood vessel walls to the liver for excretion[57, 58]. Additionally, activated LXR/RXR can reduce the expression of pro-inflammatory cytokines (e.g., IL-6, IL-1β) by inhibition of the activity of transcription factor NF-κB[59]. The acute phase response which is activated during aging and severe sepsis can inhibit LXR/RXR activation[60]. Failed activation of the LXR/RXR complex may lead to atherosclerosis[61, 62], which is associated with severe sepsis[63, 64]. Apolipoproteins may have various biological functions by forming different lipoproteins [e.g., low density lipoproteins (LDL) and high density lipoproteins (HDL)]. For example, Apo B-100 is the protein component for LDL which is a cholesterol transporter[65]. Whereas HDL formed by Apo A and Apo CIII can inhibit oxidation and inflammation[66].

Altered levels of apoliproteins have been reported in sepsis. For example, elevated levels of ApoB-100[67] and lower concentrations of Apo CIII in response to inflammation[68] have been reported. Consistent with this, we observed increased expression of ApoB-100 (1.523±0.253) and decreased expression of Apo CIII (0.690±0.294) in younger adults who developed severe sepsis. These changes may suggest suppressed activation of the LXR/RXR pathway and subsequent higher risk of atherosclerosis in younger adults (although we do not have data to support this notion). Additionally, Apo CIII has different isoforms based on the number (i.e., 0, 1, 2, and 3) of sialic acids per protein: Apo CIII-0, Apo CIII-1, Apo CIII-2, and Apo CIII-3[69]. The Apo CIII isoform ratios have been demonstrated to be correlated with mortality in younger severe sepsis patients (∼ 49 years old)[69]. For example, the ratio of Apo CIII-2/Apo CIII-1 higher than 1.25 may indicate increased risk of death in the younger severe sepsis patients[69]. ApoE also has different isoforms: Apo E2, Apo E3, Apo E4. It has been shown that the levels of Apo E2 correlate positively with that of factor VIII, which is necessary for blood clotting[70]. Also, higher levels of Apo E3 may reduce the risk of severe sepsis[70]. In this study, higher levels of Apo E (2.105±0.301) were found in younger adults consistent with a rat model[71], however lower levels (0.646±0.170) were detected in older adults in our study. Also, higher levels of Apo CI (2.165±1.022) and Apo M (1.438±0.483) were detected in older adults who developed severe sepsis. Although Apo CI levels correlate with the survivorship in ∼25 year old severe sepsis patients[72], higher concentration of Apo

CI has been reported to promote atherosclerosis[73, 74]. Apo M has also been reported to have lower concentrations in young adults severe sepsis patients [75, 76] and contributes to the anti-inflammatory response in sepsis[76]. Higher levels of Apo M in older adults who developed severe sepsis in our studies suggest that these patients have a reduced inflammatory state during acute response. That lipid metabolism is altered in these studies points to atherosclerosis as a contributing factor of severe sepsis incidence; however further analyses are necessary to support this hypothesis.

Other pathways

Other key pathways identified in these studies such as IL-12 and IL-6 signaling support a hypoinflammatory response in older adults (Table S4, Table 2)[77].Enhanced production of nitric oxide (NO) and reactive oxygen species (ROS) in macrophages has been previously reported in aging and sepsis[78, 79]. We detect differentially-expressed proteins involved in the production of NO and ROS (i.e., retinol binding protein 1, lysozyme C and clusterin) which support elevated oxidative stress in the elderly[79, 80] and severe sepsis patients[78, 81].

The nested-case control study design employed herein has identified many potential factors that may contribute to higher incidence of severe sepsis in adults older than 65 years old. There is a substantial role of inflammation and coagulation processes in acute host response and our proteomics results suggest that older individuals have hypoinflammatory and reduced coagulation responses. While other factors such as lipid metabolism and production of ROS are implicated, additional experiments are necessary in order to determine better the effects of age on these processes. For example, it would be worthwhile in future studies to measure oxidative stress markers in this CAP cohort and to examine the correlation between incidence of severe sepsis and age with atherosclerosis. A limitation to these studies is the small sample populations (N=10) used to measure a wide range of proteins that are known to vary substantially in plasma tissue[82]. Future studies include selecting key proteins to follow their expression levels in a larger cohort using an independent method, such as ELISA.

Conclusions

The incidence and mortality of severe sepsis increases with aging, especially after 65 years. This study investigated the effects of aging on initial host response to sepsis in CAP patients who eventually develop severe sepsis after hospitalization. Using a proteomics approach, several altered biological pathways were identified. Acute phase response and coagulation were highly represented in these studies, and novel pathways such as lipid metabolism, atherosclerosis, and production of NO and ROS were observed. Interestingly, differentially-expressed proteins involved in these pathways show opposite expression levels of change dependent on patient age (i.e., 50-65 and 70-85 years old). These findings provide more insight to factors that may explain higher risk, increased incidence, and mortality in older adults with severe sepsis. Future studies on a larger cohort of patients (e.g., N>50) will be performed to further understand our results and to assess proteins with low concentrations and important biological roles (e.g., acute phase response, coagulation, and lipid metabolism). Such insight will be helpful for the development of age-specific and personalized severe sepsis treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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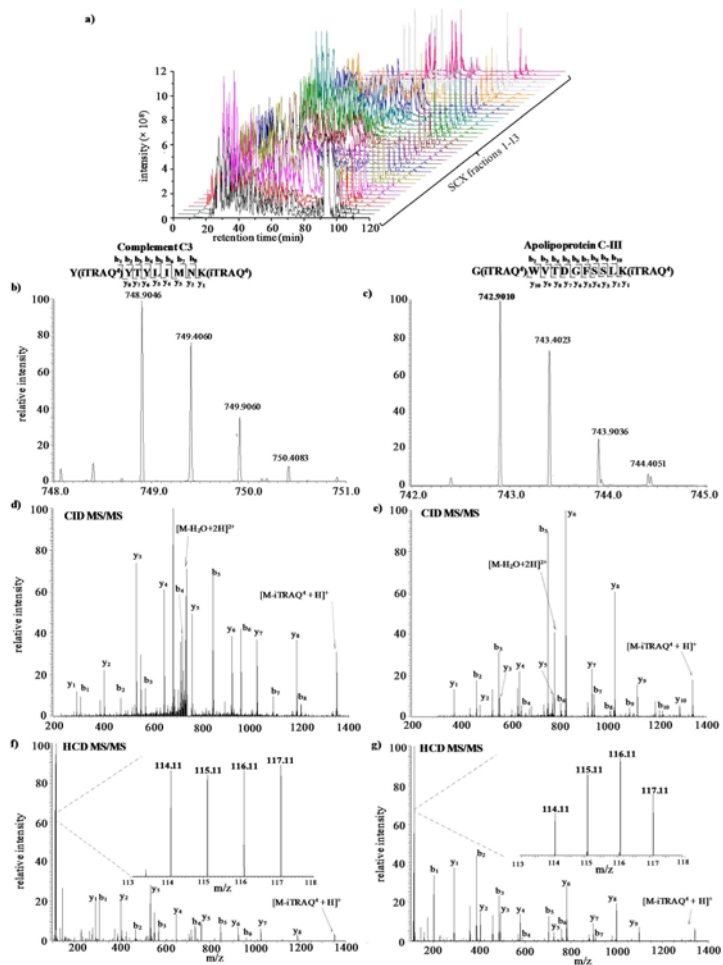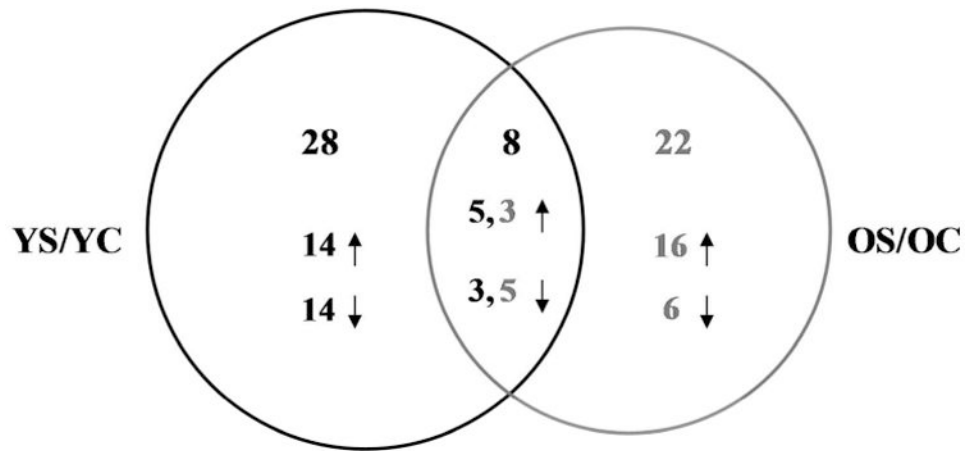
Figure 1.
a) LC chromatograms for individual SCX fractions analyzed in triplicate. Example mass spectra of peptides eluted from **b)** SCX fraction 7 t_r = 57.20 min with *m/z* 748.8046 and c) SCX fraction 6 t_r= 37.26 min with *m/z* 742.9010. CID MS/MS spectra are shown in **d)** and **e)**, respectively. HCD MS/MS are shown in **f)** and **g)**. The inserts are zoom-ins of the **f)** and **g)** low *m/z* region.
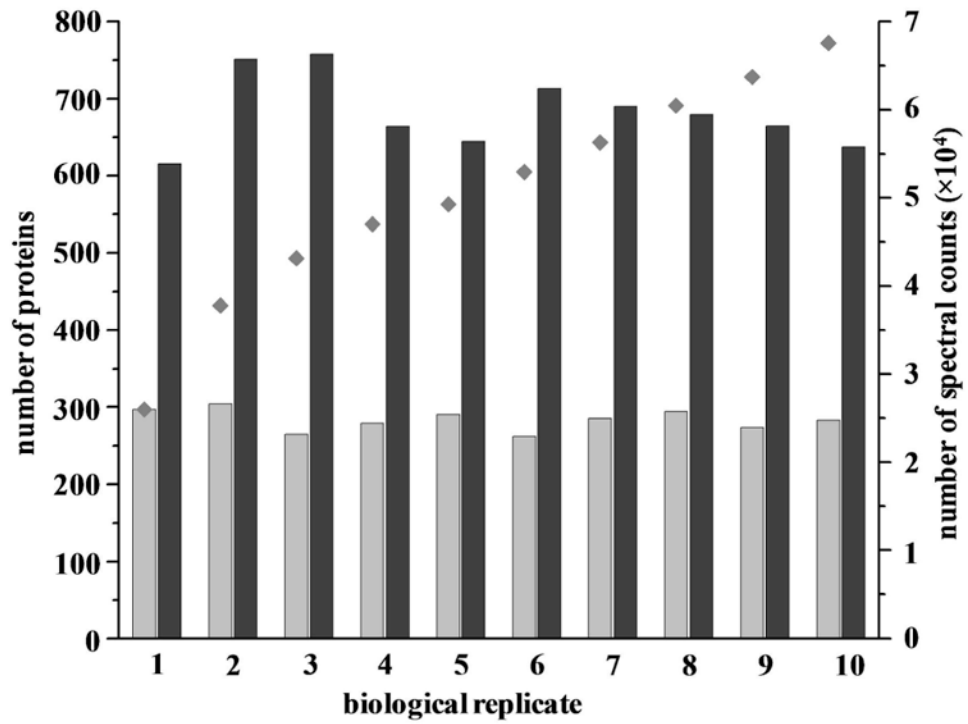
Figure 2.
The number of proteins (▨) and spectral counts (▪) identified in each experiments. The cumulative number of proteins identified with each subsequent experiment (◆) is also shown.
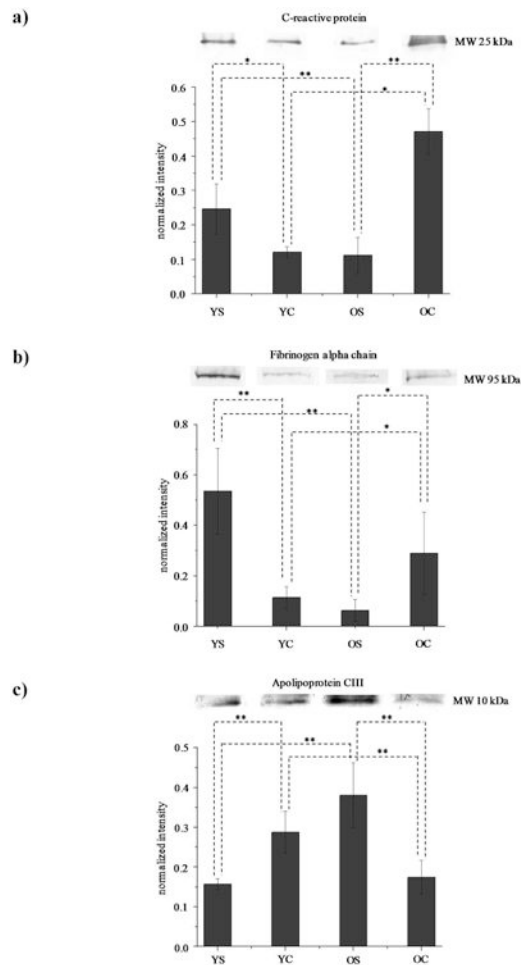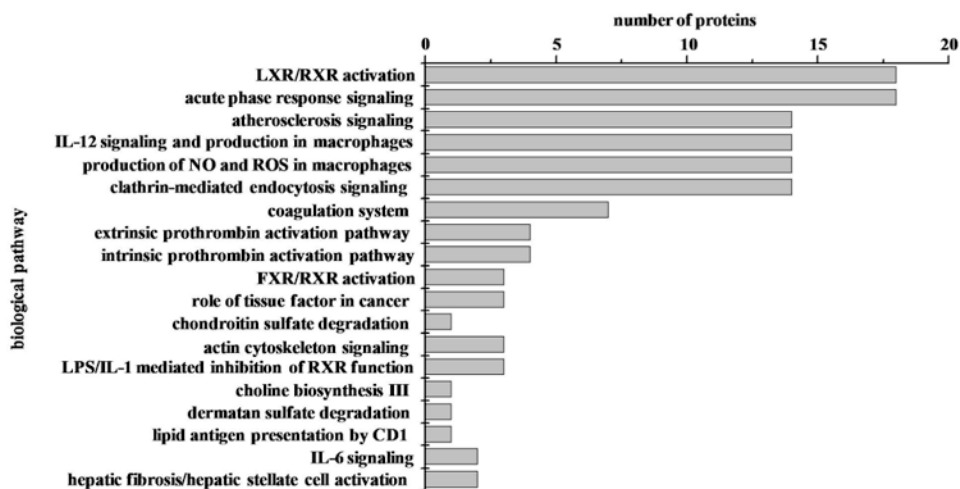
Figure 3.
Western blotting images for a) C-reactive protein, b) fibrinogen alpha chain, and c) Apo CIII. The histogram under the images displays the normalized intensity ± standard deviation (n=6) of the proteins across each group. The intensity for each individual band is normalized to the total intensity of the blot.

Figure 4.
Venn diagram of differentially-expressed proteins for each age group (i.e., YS/YC 50-65 years old and OS/OC 70-85 years old). The number of proteins that have higher (↑) or lower (↓) fold-change values in each comparison are also shown.

Figure 5.
Histogram plot of biological pathways associated with differentially expressed proteins as a function of severity of sepsis (N=58). The p value cutoff for the IPA pathways is p<0.05.

Table 1

Experimental design and iTRAQ quantitation channel assignment

Experiment	Reporter ions			
	114	**115**	**116**	**117**
1	YS[a]	YC[b]	OS[c]	OC[d]
2	YS	YC	OS	OC
3	YS	YC	OS	OC
4	YS	YC	OS	OC
5	YC	OS	OC	YS
6	YC	OS	OC	YS
7	YC	OS	OC	YS
8	OS	OC	YS	YC
9	OS	OC	YS	YC
10	OS	OC	YS	YC

[a] Patients 50-65 years old who developed severe sepsis.

[b] Patients 50-65 years old who did not develop severe sepsis.

[c] Patients 70-85 years old who developed severe sepsis.

[d] Patients 70-85 years old who did not develop severe sepsis.

Table 2

The list of proteins which are differently expressed between groups.

Acc. No.[a]	Protein Name	YS/YC (mean ± SD[b])	OS/OC (mean ± SD)	n[c]
O14791	Apolipoprotein L1	/	1.39 ± 0.70	10
O95445	Apolipoprotein M	/	1.44 ± 0.48	8
P01008	Antithrombin-III	0.60 ± 0.06	/	10
P01011	Alpha-1-antichymotrypsin	1.58 ± 0.87	0.68 ± 0.18	10
P02649	Apolipoprotein E	2.11 ± 0.30	0.65 ± 0.17	10
P02652	Apolipoprotein A-II	0.50 ± 0.08	1.45 ± 0.46	10
P02654	Apolipoprotein C-I	/	2.17 ± 1.02	10
P02656	Apolipoprotein C-III	0.69 ± 0.29	1.43 ± 0.77	10
P02671	Fibrinogen alpha chain	2.44 ± 0.72	0.60 ± 0.13	10
P02675	Fibrinogen beta chain	2.10 ± 0.58	/	10
P02679	Fibrinogen gamma chain	2.08 ± 0.72	/	10
P02735	Serum amyloid A protein	3.06 ± 1.09	/	10
P02741	C-reactive protein	3.27 ± 1.07	0.52 ± 0.22	10
P02749	Beta-2-glycoprotein 1	/	1.58 ± 0.61	10
P02750	Leucine-rich alpha-2-glycoprotein	2.14 ± 0.30	/	10
P02751	Fibronectin	/	0.62 ± 0.31	10
P02753	Retinol-binding protein 4	0.53 ± 0.09	/	10
P02763	Alpha-1-acid glycoprotein 1	2.03 ± 0.43	/	10
P02766	Transthyretin	0.44 ± 0.09	/	10
P02774	Vitamin D-binding protein	0.57 ± 0.08	/	10
P02775	Platelet basic protein	/	0.76 ± 0.21	9
P04114	Apolipoprotein B-100	1.52 ± 0.25	/	7
P04259	Keratin, type II cytoskeletal 6B	0.52 ± 0.06	/	7
P04275	von Willebrand factor	2.05 ± 0.34	/	10
P04278	Sex hormone-binding globulin	0.70 ± 0.26	/	7
P05090	Apolipoprotein D	/	1.47 ± 0.32	10
P05154	Plasma serine protease inhibitor	0.73 ± 0.46	/	6
P05452	Tetranectin	0.64 ± 0.10	/	10
P05546	Heparin cofactor 2	0.71 ± 0.26	/	10
P06276	Cholinesterase	/	1.45 ± 0.47	9
P06396	Gelsolin	0.55 ± 0.10	/	10
P07996	Thrombospondin-1	/	0.65 ± 0.34	6
P10909	Clusterin	0.73 ± 0.30	/	10
P13645	Keratin, type I cytoskeletal 10	0.58 ± 0.06	/	10
P15169	Carboxypeptidase N catalytic chain	1.31 ± 0.36	/	10
P16070	CD44 antigen	1.62 ± 0.55	/	7
P18428	Lipopolysaccharide-binding protein	2.20 ± 0.50	0.76 ± 0.32	10
P19652	Alpha-1-acid glycoprotein 2	2.17 ± 0.60	/	10
P19823	Inter-alpha-trypsin inhibitor heavy chain H2	/	1.40 ± 0.53	10

Acc. No.[a]	Protein Name	YS/YC (mean ± SD[b])	OS/OC (mean ± SD)	n[c]
P19827	Inter-alpha-trypsin inhibitor heavy chain H1	/	1.58 ± 0.58	10
P20851	C4b-binding protein beta chain	1.39 ± 0.46	/	6
P29622	Kallistatin	/	1.69 ± 0.57	10
P35542	Serum amyloid A-4 protein	0.48 ± 0.04	/	10
P35858	Insulin-like growth factor-binding protein complex acid labile subunit	/	1.39 ± 0.46	9
P61626	Lysozyme C	1.91 ± 0.96	/	8
P61769	Beta-2-microglobulin	1.89 ± 1.02	/	6
P80108	Phosphatidylinositol-glycan-specific phospholipase D	0.39 ± 0.06	/	9
Q03591	Complement factor H-related protein 1	1.31 ± 0.60	/	10
Q04756	Hepatocyte growth factor activator	/	1.81 ± 0.68	10
Q15431	Synaptonemal complex protein 1	/	1.37 ± 0.62	6
Q6UXB8	Peptidase inhibitor 16	/	2.03 ± 0.78	6
Q86UD1	Out at first protein homolog	/	0.74 ± 0.19	6
Q86UV6	Tripartite motif-containing protein 74	/	1.85 ± 0.79	6
Q96KN2	Beta-Ala-His dipeptidase	/	2.06 ± 0.93	6
Q96PD5	N-acetylmuramoyl-L-alanine amidase	0.58 ± 0.06	1.35 ± 0.39	10
Q9BXR6	Complement factor H-related protein 5	/	0.72 ± 0.18	10
Q9NXD2	Myotubularin-related protein 10	/	1.78 ± 0.69	7
Q9Y6R7	IgGFc-binding protein	/	0.70 ± 0.26	9

[a] accession number provided from the uniprot human database (04/25/2010, 20295 sequences).

[b] mean and SD values are calculated based on the reporter ion ratios for proteins quantified in at least 6 experiments.

[c] the number of experiments in which the corresponding proteins are quantified.