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Role of Heat Shock Protein and Cytokine Expression as Markers of Clinical Outcomes in GLN-Supplemented Parenteral Nutrition (GLND) Trial in Surgical ICU Patients

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Abstract

Background: Nutrients, such as glutamine (GLN), have been shown to effect levels of a family of protective proteins termed heat shock proteins (HSPs) in experimental and clinical critical illness. HSPs are believed to serve as extracellular inflammatory messengers and intracellular cytoprotective molecules. Extracellular HSP70 (eHSP70) has been termed a chaperokine due to ability to modulate the immune response. Altered levels of eHSP70 are associated with various disease states. Larger clinical trial data on GLN effect on eHSP expression and eHSP70's association with inflammatory mediators and clinical outcomes in critical illness are limited.

Objective: Explore effect of longitudinal change in serum eHSP70, eHSP27 and inflammatory cytokine levels on clinical outcomes such as pneumonia and mortality in adult surgical intensive care unit (SICU) patients. Further, evaluate effect of parenteral nutrition (PN) supplemented with GLN (GLN-PN) versus GLN-free, standard PN (STD-PN) on serum eHSP70 and eHSP27 concentrations.

Methods: Secondary observational analysis of a multicenter clinical trial in 150 adults after cardiac, vascular, or gastrointestinal surgery requiring PN support and SICU care conducted at five academic medical centers. Patients received isocaloric, isonitrogenous PN, with or without GLN dipeptide. Serum eHSP70 and eHSP27, interleukin-6 (IL-6), and 8 (IL-8) concentrations were analyzed in patient serum at baseline (prior to study PN) and over 28 days of follow up.

Results: eHSP70 declined overtime in survivors during 28 days follow-up, but non-survivors had significantly higher eHSP70 concentrations compared to survivors. In patients developing pneumonia, eHSP70, eHSP27, IL-8, and IL-6 were significantly elevated. Adjusted relative risk for hospital mortality was reduced 75% (RR = 0.25, p=0.001) for SICU patients with a faster decline in eHSP70. The area under the receiver operating characteristic curve was 0.85 (95% CI: 0.76 to 0.94) for the final model suggesting excellent discrimination between SICU survivors and non-survivors. GLN-PN did not alter eHSP70 or eHSP27 serum concentrations over time compared to STD-PN.

Conclusion: Our results suggest that serum HSP70 concentration may be an important marker for severity of illness and likelihood of recovery in the SICU. GLN-supplemented-PN did not increase eHSP70.

Keywords

parenteral nutrition; glutamine; heat shock proteins; hospital-acquired infection; critical care

Background:

HSPs are a family of conserved proteins that play key roles during stress and illness [1, 2]. They are found in cellular compartments, on the surface of cells, and in serum. HSPs are classified according to molecular weight and are well known for both their role as intracellular chaperones and extracellular modulators of the immune response [1, 2]. Extracellular HSP70 (eHSP70) has been linked to specific inflammatory states such as arthritis[3], asthma[4], diabetes[5], chronic obstructive pulmonary disease (COPD)[6], preeclampsia [7], cognitive decline in the elderly [8], cardiac hypertrophy [9]. In contrast, low levels persist in atherosclerosis [10, 11], aging [12, 13], and obesity [14]. Decreased blood levels of eHSP27 have been linked to atherosclerosis and acute coronary events [11, 15, 16]. Specifically, earlier studies have shown elevated levels of serum eHSP70 are correlated with better outcomes in a surgical ICU patient population [17], and in acute trauma [18]. However, to our knowledge there is no data correlating levels of eHSP27 with outcomes in critical illness. Both eHSP70 and eHSP27 appear to serve as immune modulators. In fact, eHSP70 has been coined a “chaperokine” in reference to its ability to chaperone proteins and induce immune signaling [19]. eHSP70 has been shown to induce production of multiple inflammatory cytokines such as tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6), IL-1 β , and IL-8 through the receptors CD14, TLR2, and TLR4, respectively [20–22]. Additionally, eHSP27 has been shown to modulate the immune system via interactions with TLR2 and TLR4 [23], signaling through NF κ B [23, 24], and inducing secretion of IL-10, GM-CSF, IL-6, TNF- α , IL-12p35, and IL-12p40 [23–25]. While there appears to be a role for HSP70 in ICU outcome, its role as a chaperokine immune system effects in critical illness remains unknown.

Current data suggests that supplementation with the amino acid Glutamine (GLN) can affect both intracellular and extracellular Heat Shock Protein (eHSP) levels in animal models and critically ill patients [17, 26–28]. Glutamine (GLN), may have important implications in critically ill patients as low GLN levels have been associated with poor critical illness outcome [29]. Previous data from our group and others has indicated enhanced HSP expression may be a key mechanism by which GLN confers protection in critical illness [17, 26–28].

GLN has been labeled a “conditionally essential” amino acid during prolonged critical illness, which led to the hypothesis that GLN supplementation would improve outcomes [30]. However, recently signals of risk of harm have come from two large-scale multicenter trials evaluating mortality utilizing a combination of high-dose intravenous/enteral GLN, the REDOXs study [31] or high-dose enteral mixture of different nutrients including GLN, the METAPLUS trial [32]. These new trials were both targeted to investigate GLN (and other nutrients) as primary pharmaconutrients, and not as supplementation to PN. These data show that unstable patients in the early phase of sepsis, on vasopressors, or in renal failure without dialysis should not get supplemental GLN. Three recent meta-analyses [33], [34, 35] have confirmed traditional PN-supplementation with intravenous GLN is safe, reduces mortality and LOS, and improves outcome. Patients in need of PN, patients with burns, trauma or malignancies, may continue to benefit from supplemental GLN, administered either

intravenously at < 0.35 g/kg/d or enterally at < 0.5 g/kg/d [36, 37] and large trials of enteral GLN supplementation in the burn setting is ongoing [38].

Our group recently published a prospective, randomized, controlled, double blind, parallel-group, intent-to-treat, Phase III multicenter study designed to define the safety and clinical efficacy of glutamine-supplemented PN in SICU patients. Unlike previous studies, we did not find a protective effect of GLN supplementation, but did demonstrate safety [39]. Thus, with conflicting risk and benefit data for GLN in critical illness, a greater understanding of GLN's potential mechanistic effects in ICU patients is needed.

The purpose of this study was to determine if eHSP and inflammatory cytokine expression is associated with key clinical outcomes such as pneumonia and mortality risk in SICU patients. Further, we evaluated if GLN-supplemented PN exerted an effect on serum eHSP levels over time compared to GLN-free PN in the context of the recent randomized clinical trial [40].

Methods:

Informed Consent

The Institutional Review Boards of Emory University (Atlanta, GA), Vanderbilt University (Nashville, TN), Miriam Hospital (Providence, RI), University of Wisconsin-Madison (Madison, WI), and University of Colorado (Aurora, CO) participated in this study ([Clinicaltrials.gov](https://clinicaltrials.gov) identifier: NCT00248638). Consent was obtained from all patients or their legally authorized representatives. Patients were then randomized after sight-specific approved consent forms were signed.

Study Coordination and Oversight

The study was coordinated as described previously by Ziegler et. al [39]. This NIDDK-funded intent-to-treat Phase III, multi-center, double-blind, placebo-controlled trial was designed to determine the effect of parenteral GLN dipeptide on important clinical outcomes (hospital mortality rate and percentage of subjects without new hospital infections) in patients requiring SICU care and PN after cardiac, vascular or intestinal surgery. Study patients were randomized to receive the dipeptide alanyl-glutamine-supplemented (GLN-PN) or isonitrogenous, isocaloric GLN-free PN (STD-PN) until enteral feeding was established. This trial was based on our pilot study which demonstrated a marked decrease in nosocomial infection, improved indices of organ function, and a possible decrease in hospital mortality in SICU patients receiving GLN-PN versus standard STD-PN [39]. A specific aim of this study was to obtain mechanistically relevant observational data on whether GLN-PN affects serial blood concentrations of heat shock proteins 70 and 27, and pro-inflammatory cytokine expression.

Study design

Adults clinically deemed to require SICU care and PN 2 to 14 days after cardiac, vascular, or intestinal surgery were eligible for the study. Subjects were randomized on APACHE II score (≤ 15 or > 15) to either the experimental GLN-PN group or the STD-PN control group

as outlined previously. Unblinded PN pharmacist managers at each clinical center maintained 2 color-coded sets of sealed, sequenced, opaque envelopes containing the treatment assignment [39].

Inclusion criteria:

Patients of at least 18 but not more than 90 years of age at time of surgery with a body mass index (BMI) < 40 kg/m² prior to surgery, 14 days postoperative from one of the following open surgical procedures: CABG, cardiac valve, vascular (non-neurosurgical), or esophago-gastrointestinal resection of esophagus, stomach, small bowel, colon and/or rectum), or operation to identify the source of peritonitis when there is evidence of a bowel perforation (with or without bowel resection) were included. Patients had to require SICU care and have been deemed to require central venous PN for at least 7 subsequent days after entry on a clinical basis. The patient's primary physician(s) allowed the investigative team to manage the study PN and enteral feedings during the current hospitalization.

Exclusion Criteria:

Pregnancy; clinical sepsis (defined as unstable blood pressure despite vasopressor support with mean arterial pressure (MAP) < 60 mm Hg on at least 3 consecutive readings within a 3-hour period during the 24 hours prior to study entry; current malignancy requiring surgery as the GLND qualifying operation or patients that received an active regimen of chemotherapy and/or radiotherapy to treat a previously diagnosed malignancy; patient history of seizures or pre-existing seizure disorder; current encephalopathy; patients with known history of cirrhosis or a serum total bilirubin level > 10.0 mg/dL; patient history of chronic renal failure requiring dialysis; or significant renal dysfunction (defined as serum creatinine > 2.5 mg/dL and not receiving continuous renal replacement therapy (CRRT) or the patient requires acute hemodialysis postoperatively; patients with a concomitant burn or trauma injury; patients who had previously undergone an organ transplantation; patients with a history of HIV/AIDS; patients who had received any investigational drug within 60 days prior to study entry; and patients who had received enteral or parenteral enteral feedings enriched in arginine and/or glutamine within 30 days prior to study entry[39].

All infections were adjudicated by an Infectious Disease expert Emory faculty member coinvestigator in a double-blind manner (HMB), as previously outlined in detail in the parent report [40].

Study Procedures Overview

Patients were followed for a total of 6 months after entry. Blood samples to analyze levels serum heat shock protein and inflammatory cytokines were obtained at entry (day 1, before initiation of study PN) and serially, when possible, on study days 3, 7, 14, 21, and 28 after entry. Patients were contacted via telephone 2, 4, and 6 months after randomization to determine vital status and whether they had been re-hospitalized or readmitted to the SICU.

Blood was collected between 8 and 10 am for each scheduled draw. A total of 148 of the randomized 150 subjects were analyzed for serum HSP-70 and HSP27 levels using all available data collected over the first 28 days on study. Whole blood was obtained for serum

HSP-70 and HSP-27 concentrations in a speckled red top vacutainer tube containing SST™ Gel and Clot Activator (BD Vacutainer®, Becton, Dickinson and Co., Franklin Lakes, NJ) at baseline. Serum samples were allowed to clot at room temperature for 30 minutes, prior to centrifugation, and then spun at 3,000 rpm for 15 minutes in a refrigerated centrifuge at 4°C. Serum heat shock protein and cytokine analysis was performed using commercially available solid-phase enzyme immunoassay (ELISA) kits. The Bio-Plex System Luminex XMAP Technology with Bio-Plex manager 3.0 software was used for analysis (Bio-Rad Laboratories Inc. Hercules, CA). Collected and processed blood samples were immediately frozen and stored at -80 degrees C and all transfers were done on dry ice. Samples were analyzed in batches in close time-proximity to collection. We have previously shown this is a reliable method to determine eHSP and cytokine expression [17].

Statistical Analysis

The statistical test for interaction between time on study and treatment was the primary overall hypothesis test to determine whether an outcome in the two study groups changed in significantly different ways during follow-up (i.e., different temporal patterns over time). If a mean outcome in the two treatment groups was consistently different or similar over time (i.e., no statistical interaction) then the main effect test for treatment was used as the primary test. If a significant interaction was detected, then t-tests were used to compare the differences between the model-based treatment means at each time point and to compare differences over time within each treatment arm. Specific statistical tests were done within the framework of the mixed effects linear model. All statistical tests were 2-sided and unadjusted for multiple comparisons. This same model was used to compare outcomes by hospital survival status, pneumonia and blood stream infection diagnoses.

After analysis, the HSP-70 mean and its 95% confidence interval (CI) were back transformed to the original scale and reported as the geometric mean with 95% CI. Similarly, back transformation of the difference between the mean of log-transformed HSP-70 among GLN-PN treated SCIU patients and control (STD-PN) SICU patients yielded the ratio of the geometric means. Confidence intervals for the geometric mean ratio (GMR) were computed by back transforming the 95% confidence bounds for the mean difference. A GMR of 1.0 suggests no treatment effect. The geometric mean and its 95% CI and the GMR and its 95% CI were also calculated for IL-6 and IL-8.

Repeated-measures analyses using mixed linear models were performed for HSP-70, HSP-27, IL-6 and IL-8 concentrations over time. The analyses were done with a means model via the SAS MIXED Procedure (version 9.4; SAS Institute, Cary, NC) providing separate estimates of the means by time on study (0, 3, 7, 14, 21 and 28 days) and treatment group. A natural log transformation was performed prior to the HSP-70, IL-6 and IL-8 analyses. The log transformation improved the symmetry in the outcome data. A data transformation was not necessary for HSP-27.

For each of the four serum outcomes, the statistical model included three predictors (treatment arm, time on study (categorical clinical visit) and the statistical interaction between treatment arm and time on study. A compound-symmetric variance-covariance form in repeated measurements was assumed for each outcome and robust estimates of the

standard errors of parameters were used to perform statistical tests and construct 95% confidence intervals [40]. The model-based means are unbiased with unbalanced and missing data, so long as the missing data are non-informative (missing at random, MAR). A P-value 0.05 was considered statistically significant for the main effects (treatment and time on study) and for the treatment by time on study interaction effect from the repeated-measures analysis.

Rates of change of HSP-70 were obtained by using a mixed-effects linear model specifying that HSP-70 measurements follow a linear regression over levels of IL-8 (and separately for IL-6), with a random intercept and slope for each patient. The mean slope was estimated and compared by hospital mortality and by pneumonia diagnosis during follow-up (yes or no).

Two approaches were used to evaluate eHSP70 as a “biomarker” of patient outcome. First, the risk of each patient outcome was modeled as a function eHSP70 by using a modified Poisson regression model. Second, biomarker performance was summarized with classification performance measures, such as sensitivity, specificity and receiver operating characteristic (ROC) curves. Additional details for these two approaches are described in the next two paragraphs.

Relative risks were calculated to measure the degree of association between risk factors and hospital mortality by fitting a modified Poisson regression model separately for each risk factor. The modified Poisson regression model is appropriate for a binary outcome when the error for the estimated relative risk is estimated using a robust error variance known as a sandwich estimator. This methodology and the implementation using the SAS Genmod Procedure (version 9.4, generalized linear models) are described by Zou [41]. Due to the limited number of patients who died in the hospital and concern with model overfitting, the number of covariates included in multivariable modified Poisson regression analysis was limited to the main effects for the following six covariates: Apache II score at the time of ICU admission, age, mechanical ventilation at the time of study entry, baseline IL-6, baseline IL-8 and the estimated linear slope for HSP70. Covariate selection was driven by available clinical knowledge and biological plausibility of potential confounders.

Apache II score, and IL-6 and IL-8 serum concentrations were not included in the multivariable regression analyses since these covariates were not statistically associated with hospital mortality in univariable analysis. The relative risk and its 95% confidence interval were calculated for each risk factor in the presence of others in the final model. The predictive power or predictive ability of the final regression model to discriminate between survivors and non-survivors was quantified by the area under the receiver operator characteristic curve and its 95% confidence interval. These same analyses were also performed for the outcome incident pneumonia, incident BSI and the composite outcome of hospital mortality or pneumonia. Please see supplemental methods for additional statistical details.

Results:

GLN Dipeptide Supplemented PN did not increase serum HSP70 concentrations

In this study, we did not observe a difference in serum HSP70 expression between the GLN-PN and GLN-free PN groups (Figure 1A). Mean HSP70 levels in serum were similar between the two treatment groups pooled across days on study (model-based geometric means = 27.0 ng/mL for GLN-PN and 25.2 ng/mL for STD-PN, $P = 0.62$). Serum HSP70 levels declined in similar ways in the two treatment groups over time during follow-up ($p=0.53$, test for interaction between days on study and treatment group). However, the decline over time in serum HSP70 was statistically significant. The geometric mean for HSP70 declined over the 28 days in each of the two treatment groups ($P < 0.001$ and $P < 0.001$ for GLN-PN and STD-PN respectively) (Figure 1A).

Serum HSP70 levels were increased in non-survivors

To determine if serum HSP70 levels were associated with 28-day hospital mortality, we assessed the difference between survivors and non-survivors. Non-survivors had increased levels of serum HSP70 over time compared to survivors (Figure 2A and Supplemental Figure 1). The levels of HSP70 changed in significantly different ways during follow-up between the two groups ($P < 0.001$ test for interaction between days on study and vital status group). Figure 2A shows the mean serum HSP70 concentration was similar in both groups at the time of randomization ($P = 0.14$) but became significantly higher in the non-survivors at 3 days (55.6 ng/mL vs 28.8 ng/mL, $P = 0.0018$), 7 days (58.7 ng/mL vs 26.4 ng/mL, $P = 0.0002$), 14 days (51.1 ng/mL vs 20.7 ng/mL, $P < 0.0001$), 21 days (62.2 ng/mL vs 18.0 ng/mL, $p < 0.0001$) and 28 days (48.8 ng/mL vs 16.2 ng/mL, $P < 0.0001$). At 14 days, serum HSP70 levels were 2.47-fold higher in non-survivors compared to survivors, with a GMR of 2.47 (95% CI: 1.64 – 3.72, $p < 0.001$). Further, using a mixed-effects linear model, the slopes for the rate of change in HSP70 over time were significantly different. Supplemental Figure 1 summarizes the average rate of change (slope) for survivors (slope $\pm$ SE: -0.02836 ± 0.002105 , $p < 0.001$; intercept $\pm$ SE: 3.4876 ± 0.06925) and non-survivors (slope $\pm$ SE: 0.006586 ± 0.006586 , $p = 0.29$; intercept $\pm$ SE: 3.9231 ± 0.1627).

Serum HSP70 levels were increased in patients diagnosed with new hospital-acquired pneumonia but not in those with new bloodstream infections.

We further examined serum HSP70 levels in patients with major hospital-acquired infections. Specifically, we compared the levels in patients who developed new hospital-acquired pneumonia (Figure 3A) or new hospital-acquired bloodstream infections (BSI; Figure 4A) compared to patients who did not have such documented infections. The levels of HSP70 changed in significantly different ways during follow-up between the two groups ($P < 0.001$ test for interaction between days on study and pneumonia). When pooled across time on the study, patients diagnosed with pneumonia had significantly higher HSP70 concentrations (geometric mean = 38.1 ng/ml) compared to patients that did not develop pneumonia (geometric mean = 23.9 ng/ml; $P = 0.0024$). Patients diagnosed with incident pneumonia had significantly higher (1.9 fold) serum HSP70 concentrations compared to patients without incident pneumonia (GMR = 1.88, 95% CI: 1.35–2.62, $P = 0.0002$ at day 14

of follow-up). However, we did not observe a significant difference in HSP70 levels between patients with bloodstream infection (BSI) and those without a BSI (Figure 4A).

Serum HSP27 was not increased with GLN supplemented PN or in survivors

Serum HSP27 has also been associated with disease but has not been examined in a critically ill population. GLN supplemented PN did not affect serum levels of HSP27 over time (Figure 1B). The levels of HSP27 were similar during follow-up for the two treatment groups. Serum HSP27 levels in 28-day survivors and non-survivors were similar ($p = 0.46$, test for interaction between days on study and mortality status group) (Figure 2B). However, mean serum HSP27 levels were increased in patients with pneumonia vs. no pneumonia pooled across days on the study (Figure 3B). Mean HSP27 levels were similar between patients with incident BSI compared to patients without BSI ($P = 0.37$, Figure 4B).

Patients with pneumonia had significantly higher levels of IL-8 and IL-6

There was no difference in levels of IL-8 (Figure 1C) and IL-6 levels (Figure 1D) in patients who received glutamine supplementation in PN versus patients who did not. Further, there was not a significant difference in serum levels of IL-8 (Figure 2C) or IL-6 (Figure 2D) between non-survivors vs. survivors. There were significantly higher levels of IL-8 (Figure 3C) and IL-6 (Figure 3D) in patients with pneumonia but not with BSI (Figures 4C and Figure 4D). The serum concentration means for IL-8 pooled across day of the study in new hospital pneumonia versus no pneumonia patients were different (52.5 pg/mL versus 39.3 pg/mL; $P = 0.046$) as were the means for serum IL-6 levels pooled across days of study (92.4 pg/mL versus 52.2 pg/mL; $P = 0.0067$).

Longitudinal relationships between serum HSP70, IL-8, and IL-6 concentrations

We investigated the relationship between HSP70 and the pro-inflammatory cytokines IL-8 and IL-6. The average rate of increase in HSP70 as IL-8 increased was similar for survivors and non-survivors ($P=0.97$, Figure 5A). However mean HSP70 was significantly higher for non-survivors compared to survivors across the range of IL-8. Additionally, the average rate of increase in HSP70 as IL-8 increased was similar for SICU patients with and without incident pneumonia ($P=0.17$, Figure 5B). However mean HSP70 was significantly elevated in incident pneumonia patients compared to SICU patients who did not develop pneumonia as IL-8 increased from log 1.0 to log 3.0. (Figure 5B, $P = 0.05$, $P=0.03$, and $P=0.03$ respectively).

The slope or average rate of increase in HSP70 as IL-6 increased was different for survivors and non-survivors ($p=0.047$, Figure 5C). Mean HSP-70 was significantly higher for non-survivors compared to survivors across the range of IL-6 (Figure 5C). The slope or average rate of increase in HSP70 as IL-6 increased was not quite statistically different for SICU patients with and without incident pneumonia ($p=0.054$, Figure 5D). However, mean HSP-70 was significantly elevated in patients with pneumonia patients compared to patients who did not develop pneumonia as IL-6 increased from log 1.0 to log 4.0 (Figure 5D, $P = 0.01$, $P=0.01$, $P=0.02$ and $P=0.05$, respectively).

HSP70 decline as an independent predictor of hospital mortality and pneumonia

In univariable analysis, factors associated with hospital mortality included serum HSP70 levels at baseline and rate of HSP70 concentration decline, age at enrollment, and whether subjects were on mechanical ventilation at study entry (Table 1). The adjusted relative risk for hospital mortality was reduced 75% (RR = 0.25, $p=0.001$) for SICU patients with a faster linear decline in HSP70 (5% mortality) compared to patients with slower rate of HSP70 decline (26% mortality, Table 2). Supplemental Figure 1 demonstrates differences in slopes. The area under the receiver operating characteristic curve (AUC) for the final regression model was used to summarize the diagnostic value of the final model to discriminate between SICU survivors and non-survivors (Supplemental Figure 2). Further, a similar trend was observed in patients with pneumonia. The adjusted relative risk for pneumonia was reduced 75% (RR = 0.25, $p=0.001$) for SICU patients with a faster linear decline in HSP70 (5% with pneumonia) compared to patients with a slower rate of HSP70 decline (25% with pneumonia, Table 2).

Discussion

The results of this study demonstrate the novel finding that increased levels of serum HSP70 correlate with both mortality and pneumonia in surgical ICU patients. Further, our data show for the first time that the rate of decline of extracellular serum HSP70 levels was a predictor of both mortality and pneumonia in the ICU setting. Further, an additional novel finding is the association between HSP70 levels and the pro-inflammatory cytokines IL-6 and IL-8. To our knowledge, this is also one of the first reports of extracellular serum HSP27 levels in an ICU patient population. Increased eHSP27 levels were not correlated with mortality, but for the first time we show eHSP27, correlates with pneumonia occurrence. Interestingly, we found no association between GLN-supplemented PN and eHSP70 or eHSP27 expression.

Our current data demonstrate a relationship between increased eHSP70 levels, mortality, and pneumonia. Previous data on the relationship between levels of eHSP70 and clinical outcomes are mixed. Some studies demonstrate increased levels of eHSP70 associated with improved outcomes, while others demonstrate worse outcomes. For example, increased eHSP70 levels in trauma patients are associated with increased survival [18], but other studies indicate increased levels of serum eHSP70 are associated with increased severity if chronic disease such as diabetes, COPD, and asthma [4–6]. Interestingly, Gehrmann et al demonstrated that serum levels of HSP70 were correlated with disease severity in liver disease. Levels of eHSP70 were elevated in patients with hepatocellular cancer as compared to patients with cirrhosis. Both groups of patients had significantly higher levels than healthy controls. Additionally, a subset of patients with cirrhosis that developed liver cancer had higher serum HSP70 levels [42]. We believe it is difficult to state with certainty the importance of elevated levels of eHSP70 when looking at a single point in time.

As previously mentioned, we were able to demonstrate with univariable and multivariable analysis that the rate of decline in eHSP70 was an important predictor of mortality and pneumonia. One can hypothesize that there are three groups of patients: 1) patients that have an increase and then decrease in eHSP70 in response to stress, 2) patients that have an increase in eHSP70 that remains elevated over time in response to stress, and 3) patients that

do not increase eHSP70 in response to stress. Group 1 likely represents a beneficial response to stress, while groups 2 and 3 represent a detrimental response to stress. Group 1 is likely represented by patients who survived severe trauma in the trauma study mentioned above, since levels returned to normal 24 hours after admission [18]. Survivors in our study also fall into group 1, although we did not have a health volunteer group to definitively say that levels of eHSP70 were elevated at the start of the study. Group 2 is likely represented by the patients with cirrhosis that go on to develop cancer and the nonsurvivors [42] in our study. Group 3 is likely represented by non-survivors in the trauma study [18]. Taken together with the literature, our study presents exciting data to support the above-mentioned hypothesis. Trending levels of eHSP70 over time represents an interesting potential for a biomarker that is able to predict clinical outcomes in critically ill patients. However, further studies measuring eHSP70 levels over time are required to test above mentioned hypothesis.

Increased levels of HSP70 are associated with severe infection. In a pediatric critically ill population serum eHSP70 levels were correlated with septic shock [43]. Our data further demonstrate an association between elevated eHSP70 levels and infection, in particular pneumonia. Additionally, we show increased levels of both IL-6 and IL-8 are also associated with pneumonia. Extracellular HSP70 has been proposed to act as a “chaperokine” by modulating the immune system. Studies have shown that eHSP70 can induce production of pro-inflammatory cytokines such as TNF α , IL-6, IL-1 β , IL-8 through the receptors CD14, TLR2, and TLR4 [20–22]. Other data suggest that HSP70 induces a “regulatory” phenotype in dendritic cells, leading to induction of regulatory T cells [44, 45]. Concern over the role of LPS contamination in eHSP70 immune signaling has been raised [46], and also refuted [47]. It is possible that eHSP70 modulation of immune responses is dependent upon the level of eHSP70 as proposed by Giuliano et al, and may reflect the chaperokine ability of HSP to bind LPS, although this hypothesis has not been tested [2]. Alternatively, continued elevation in levels in eHSP70 may reflect ongoing inflammation that proves detrimental for patients.

We also examined levels of eHSP27, which, to our knowledge, has not been previously examined in critically ill patients. While we found no correlation with mortality, we did observe a correlation between increased eHSP27 levels and pneumonia at one time point, but the clinical significance of this data remains unclear. Literature indicates the level of HSP27 is correlated with disease states, in particular breast cancer, atherosclerosis, and cardiac disease. In all of these disease states decreased HSP27 is associated with better outcomes. [11, 15, 16, 48]. This may also be true in critical illness, but further studies are warranted.

In contrast to our previous pilot study [17], in this multicenter RCT GLN-supplemented PN did not lead to significant changes in eHSP70 levels. Our previously published results showed that mean plasma GLN concentrations were in the low to low-normal range at entry in both groups and levels rose significantly (by day 3) with GLN-PN [39]. GLN concentrations remained in this range and were significantly higher than in STD-PN patients through day 14. With STD-PN, plasma GLN levels rose slowly and modestly over time and plasma GLN concentrations were similar between two study groups at the days 21 and 28 time points, (reflecting transition from study PN to enteral feeds). However, plasma GLN levels in both studies were similarly elevated at day 7 in the GLN-PN group compared to the STD-PN group [17, 39]. The randomization APACHE II scores from our earlier single-

center RCT, which showed an increase in serum HSP70 with the GLN-PN were modestly lower (approximately 13 for both GLN and STD-PN groups), than in the current study (approximately 16 for both GLN-PN and STD-PN groups). It is possible in patients that are more stressed (higher APACHE score) GLN-supplemented PN is unable to exert the same effect on eHSP70. Finally, changes in ICU standards of care may play a role in GLN's effect on HSP expression, as our original pilot trial was completed prior to standards of care now in practice, such as use of glucose control and modern tidal volume ventilator management [17, 39].

A strength of our study is that it included surgical ICU patients in a randomized controlled trial at multiple (five) sites. Blood samples were collected longitudinally over a period of 28 days to track eHSP70 levels. Other studies examining the role of eHSP70 in disease have looked at only one time point [4, 7, 18]. In our patient population, we observed similar eHSP70 levels at baseline in all patients. However, during follow up, eHSP70 levels became significantly higher in non-survivors and patients diagnosed with pneumonia. The rate of decline in eHSP70 levels over time was also predictive hospital mortality and pneumonia. These assessments have not been possible in previous studies examining a single time point.

Potential limitations of this work include that the eHSP70 and eHSP27 findings may be effected by the GLN-supplementation in the study. However, as we saw no effect of GLN treatment in either eHSP70 or eHSP27 expression it is less likely that the association of eHSP70 and eHSP27 are significantly effected in this study by the GLN intervention. Unfortunately, there are not currently well-accepted normal levels for eHSPs in health or critical illness. We hope our new data will help establish a range of eHSP levels for future investigations. It also should be noted that eHSP70 and eHSP27 may not be consistently reflective of intracellular HSP levels which play a direct role in cellular protection. Future studies should be undertaken to look at the association of intracellular HSP levels and key clinical outcomes. These studies could be reasonably conducted by collecting peripheral blood mononuclear cells (PBMCs) in ICU patients and measuring intracellular HSP levels and correlating to clinical ICU outcomes and expression of inflammatory markers.

Conclusion

In conclusion, our results support a role for extracellular (serum) HSP70 as a biomarker for both mortality and pneumonia in a critically ill population. The mechanistic roles of eHSP70 in critical illness continue to evolve. The continued elevation of HSP70 may represent ongoing inflammation that proves detrimental to patients. However, the precise effect eHSP70 may have on systemic inflammation and clinical outcomes in ICU patients requires further study. Finally, our data suggest that in current ICU practice GLN-supplemented-PN did not effect eHSP70 in our patient population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Radons J: The human HSP70 family of chaperones: where do we stand? *Cell Stress Chaperones* 2016, 21(3):379–404. [PubMed: 26865365]
2. Giuliano JS Jr., Lahni PM, Wong HR, Wheeler DS: Pediatric Sepsis - Part V: Extracellular Heat Shock Proteins: Alarmins for the Host Immune System. *Open Inflamm J* 2011,4:49–60. [PubMed: 24765217]
3. Luo X, Zuo X, Zhou Y, Zhang B, Shi Y, Liu M, Wang K, McMillian DR, Xiao X: Extracellular heat shock protein 70 inhibits tumour necrosis factor- α induced proinflammatory mediator production in fibroblast-like synoviocytes. *Arthritis Res Ther* 2008,10(2):R41. [PubMed: 18410682]
4. Hou C, Zhao H, Li W, Liang Z, Zhang D, Liu L, Tong W, Cai SX, Zou F: Increased heat shock protein 70 levels in induced sputum and plasma correlate with severity of asthma patients. *Cell Stress Chaperones* 2011,16(6):663–671. [PubMed: 21643870]
5. Krause M, Heck TG, Bittencourt A, Scmazzon SP, Newsholme P, Curi R, Homem de Bittencourt PI Jr., The chaperone balance hypothesis: the importance of the extracellular to intracellular HSP70 ratio to inflammation-driven type 2 diabetes, the effect of exercise, and the implications for clinical management. *Mediators Inflamm* 2015, 2015:249205. [PubMed: 25814786]
6. Dong J, Guo L, Liao Z, Zhang M, Wang T, Chen L, Xu D, Feng Y, Wen F: Increased expression of heat shock protein 70 in chronic obstructive pulmonary disease. *Int Immunopharmacol* 2013,17(3):885–893. [PubMed: 24095952]
7. Peracoli JC, Bannwart-Castro CF, Romao M, Weel IC, Ribeiro VR, Borges VT, Rudge MV, Witkin SS, Peracoli MT: High levels of heat shock protein 70 are associated with pro-inflammatory cytokines and may differentiate early- from late-onset preeclampsia. *J Reprod Immunol* 2013,100(2):129–134. [PubMed: 24051131]
8. Son SJ, Lee KS, Chung JH, Chang KJ, Roh HW, Kim SH, Jin T, Back JH, Kim HJ, Lee Y et al.: Increased plasma levels of heat shock protein 70 associated with subsequent clinical conversion to mild cognitive impairment in cognitively healthy elderly. *PLoS One* 2015,10(3):e0119180. [PubMed: 25768018]
9. Cai WF, Zhang XW, Yan HM, Ma YG, Wang XX, Yan J, Xin BM, Lv XX, Wang QQ, Wang ZY et al.: Intracellular or extracellular heat shock protein 70 differentially regulates cardiac remodelling in pressure overload mice. *Cardiovasc Res* 2010, 88(1):140–149. [PubMed: 20542874]
10. Dulin E, Garcia-Barreno P, Guisasola MC: Extracellular heat shock protein 70 (HSPA1A) and classical vascular risk factors in a general population. *Cell Stress Chaperones* 2010, 15(6):929–937. [PubMed: 20490736]
11. Martin-Ventura JL, Leclercq A, Blanco-Colio LM, Egido J, Rossignol P, Meilhac o, Michel JB : Low plasma levels of HSP70 in patients with carotid atherosclerosis are associated with increased levels of proteolytic markers of neutrophil activation. *Atherosclerosis* 2007, 194(2):334–341. [PubMed: 17161406]
12. Jin X, Wang R, Xiao C, Cheng L, Wang F, Yang L, Feng T, Chen M, Chen S, Fu X et al.: Serum and lymphocyte levels of heat shock protein 70 in aging: a study in the normal Chinese population. *Cell Stress Chaperones* 2004, 9(1):69–75. [PubMed: 15270079]
13. Njemini R, Bautmans I, Onyema OO, Van Puyvelde K, Demanet C, Mets T: Circulating heat shock protein 70 in health, aging and disease. *BMC Immunol* 2011,12:24. [PubMed: 21443787]

14. Di Naso FC, Porto RR, Fillmann HS, Maggioni L, Padoin AV, Ramos RJ, Mottin CC, Bittencourt A, Marroni NA, de Bittencourt PI Jr., Obesity depresses the anti-inflammatory HSP70 pathway, contributing to NAFLD progression. *Obesity (Silver Spring)* 2015, 23(1):120–129. [PubMed: 25292174]
15. Jin C, Phillips VL, Williams MJ, van Rij AM, Jones GT: Plasma heat shock protein 27 is associated with coronary artery disease, abdominal aortic aneurysm and peripheral artery disease. *Springerplus* 2014, 3:635. [PubMed: 25392804]
16. Seibert TA, Hibbert B, Chen YX, Rayner K, Simard T, Hu T, Cuerrier CM, Zhao X, de Belleruche J, Chow BJ et al.: Serum heat shock protein 27 levels represent a potential therapeutic target for atherosclerosis: observations from a human cohort and treatment of female mice. *J Am Coll Cardiol* 2013, 62(16):1446–1454. [PubMed: 23764828]
17. Ziegler TR, Ogden LG, Singleton KD, Luo M, Fernandez-Estivariz C, Griffith DP, Galloway JR, Wischmeyer PE: Parenteral glutamine increases serum heat shock protein 70 in critically ill patients. *Intensive Care Med* 2005, 31(8):1079–1086. [PubMed: 15973519]
18. Pittet JF, Lee H, Morabito D, Howard MB, Welch WJ, Mackersie RC: Serum levels of Hsp 72 measured early after trauma correlate with survival. *J Trauma* 2002, 52(4):611–617; discussion 617. [PubMed: 11956372]
19. Asea A: Mechanisms of HSP72 release. *J Biosci* 2007, 32(3):579–584. [PubMed: 17536177]
20. Asea A, Kibingu E, Stevenson MA, Calderwood SK: HSP70 peptidembearing and peptide-negative preparations act as chaperokines. *Cell Stress Chaperones* 2000, 5(5):425–431. [PubMed: 11189447]
21. Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, Calderwood SK: HSP70 stimulates cytokine production through a CD 14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med* 2000, 6(4):435–442. [PubMed: 10742151]
22. Wheeler DS, Chase MA, Senft AP, Poynter SE, Wong HR, Page K: Extracellular Hsp72, an endogenous DAMP, is released by virally infected airway epithelial cells and activates neutrophils via Toll-like receptor (TLR)-4. *Respir Res* 2009, 10:31. [PubMed: 19405961]
23. Jin C, Cleveland JC, Ao L, Li J, Zeng Q, Fullerton DA, Meng X: Human myocardium releases heat shock protein 27 (HSP27) after global ischemia: the proinflammatory effect of extracellular HSP27 through toll-like receptor (TLR)-2 and TLR4. *Mol Med* 2014, 20:280–289. [PubMed: 24918749]
24. Salari S, Seibert T, Chen YX, Hu T, Shi C, Zhao X, Cuerrier CM, Raizman JE, O'Brien ER: Extracellular HSP27 acts as a signaling molecule to activate NF-kappaB in macrophages. *Cell Stress Chaperones* 2013, 18(1):53–63. [PubMed: 22851137]
25. Yusuf N, Nasti TH, Huang CM, Huber BS, Jaleel T, Lin HY, Xu H, Elmetts CA: Heat shock proteins HSP27 and HSP70 are present in the skin and are important mediators of allergic contact hypersensitivity. *J Immunol* 2009, 182(1):675–683. [PubMed: 19109201]
26. Hamiel CR, Pinto S, Hau A, Wischmeyer PE: Glutamine enhances heat shock protein 70 expression via increased hexosamine biosynthetic pathway activity. *Am J Physiol Cell Physiol* 2009, 297(6):C1509–1519. [PubMed: 19776393]
27. Peng ZY, Serkova NJ, Kominsky DJ, Brown JL, Wischmeyer PE: Glutamine-mediated attenuation of cellular metabolic dysfunction and cell death after injury is dependent on heat shock factor-1 expression. *JPENJ Parenter Enteral Nutr* 2006, 30(5):373–378; discussion 379.
28. Singleton KD, Wischmeyer PE: Oral glutamine enhances heat shock protein expression and improves survival following hyperthermia. *Shock* 2006, 25(3):295–299. [PubMed: 16552363]
29. Rodas PC, Rooyackers O, Hebert C, Norberg A, Wernerman J: Glutamine and glutathione at ICU admission in relation to outcome. *Clin Sci (Lond)* 2012, 122(12):591–597.
30. Bongers T, Griffiths RD, McArdle A: Exogenous glutamine: the clinical evidence. *Crit Care Med* 2007, 35(9 Suppl):S545–552. [PubMed: 17713407]
31. Heyland D, Muscedere J, Wischmeyer PE, Cook D, Jones G, Albert M, Elke G, Berger MM, Day AG, Canadian Critical Care Trials G: A randomized trial of glutamine and antioxidants in critically ill patients. *N Engl J Med* 2013, 368(16):1489–1497. [PubMed: 23594003]
32. van Zanten AR, Sztark F, Kaisers UX, Zielmann S, Felbinger TW, Sablotzki AR, De Waele JJ, Timsit JF, Honing ML, Keh D et al. High-protein enteral nutrition enriched with immune-

- modulating nutrients vs standard high-protein enteral nutrition and nosocomial infections in the ICU: a randomized clinical trial. *Jama* 2014, 312(5):514–524. [PubMed: 25096691]
33. Wischmeyer PE, Dhaliwal R, McCall M, Ziegler TR, Heyland DK: Parenteral glutamine supplementation in critical illness: a systematic review. *Crit Care* 2014,18(2):R76. [PubMed: 24745648]
 34. Bollhalder L, Pfeil AM, Tomonaga Y, Schwenkglenks M: A systematic literature review and meta-analysis of randomized clinical trials of parenteral glutamine supplementation. *Clin Nutr* 2013, 32(2):213–223. [PubMed: 23196117]
 35. Stehle P, Ellger B, Kojic D, Feuersenger A, Schneid C, Stover J, Scheiner D, Westphal M: Glutamine dipeptide-supplemented parenteral nutrition improves the clinical outcomes of critically ill patients: A systematic evaluation of randomised controlled trials. *Clin Nutr ESPEN* 2017, 17:75–85. [PubMed: 28361751]
 36. Vanek VW, Matarese LE, Robinson M, Sacks GS, Young LS, Kochevar M: A.S.P.E.N. position paper: parenteral nutrition glutamine supplementation. *Nutr Clin Pract* 2011, 26(4):479–494. [PubMed: 21697551]
 37. Wischmeyer P: Glutamine Supplementation in Parenteral Nutrition and Intensive Care Unit Patients: Are We Throwing the Baby Out With the Bathwater? *JPEN J Parenter Enteral Nutr* 2015,39(8):893–897. [PubMed: 26136273]
 38. Heyland DK, Wischmeyer G, Paul G, Jeschke G, Marc G, Wibbenmeyer F, Lucy F, Turgeon F, Alexis F, Stelfox T, Henry T, Day Andrew G., Garrel, Dominique for the RE-ENERGIZE Trial Team: A Randomized trial of EntERal Glutamine to minimIZE thermal injury (The RE-ENERGIZE Trial): a clinical trial protocol. *Scars, Burns, & Healing* 2017, 3:1–5.
 39. Ziegler TR, May AK, Hebbard G, Easley KA, Griffith DP, Dave N, Collier BR, Cotsonis GA, Hao L, Leong T et al. Efficacy and Safety of Glutamine-supplemented Parenteral Nutrition in Surgical ICU Patients: An American Multicenter Randomized Controlled Trial. *Ann Surg* 2016, 263(4):646–655. [PubMed: 26501700]
 40. Diggle P, Liang K-Y, Zeger SL: Analysis of longitudinal data. Oxford New York: Clarendon Press;Oxford University Press; 1994.
 41. Zou G: A modified poisson regression approach to prospective studies with binary data. *Am J Epidemiol* 2004,159(7):702–706. [PubMed: 15033648]
 42. Gehrman M, Cervello M, Montalto G, Cappello F, Gulino A, Knappe C, Specht HM, Multhoff G: Heat shock protein 70 serum levels differ significantly in patients with chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. *Front Immunol* 2014, 5:307. [PubMed: 25071768]
 43. Wheeler DS, Fisher LE Jr., Catravas JD, Jacobs BR, Carrillo JA, Wong HR: Extracellular hsp70 levels in children with septic shock. *Pediatr Crit Care Med* 2005, 6(3):308–311. [PubMed: 15857530]
 44. Motta A, Schmitz C, Rodrigues L, Ribeiro F, Teixeira C, Detanico T, Bonan C, Zwickey H, Bonorino C: Mycobacterium tuberculosis heat-shock protein 70 impairs maturation of dendritic cells from bone marrow precursors, induces interleukin-10 production and inhibits T-cell proliferation in vitro. *Immunology* 2007,121(4):462–472. [PubMed: 17346283]
 45. Stocki P, Wang XN, Dickinson AM: Inducible heat shock protein 70 reduces T cell responses and stimulatory capacity of monocyte-derived dendritic cells. *J Biol Chem* 2012,287(15): 12387–12394. [PubMed: 22334699]
 46. Tsan MF, Gao B: Heat shock proteins and immune system. *J Leukoc Biol* 2009, 85(6):905–910. [PubMed: 19276179]
 47. Henderson B, Calderwood SK, Coates AR, Cohen I, van Eden W, Lehner T, Pockley AG: Caught with their PAMPs down? The extracellular signalling actions of molecular chaperones are not due to microbial contaminants. *Cell Stress Chaperones* 2010,15(2):123–141. [PubMed: 19731087]
 48. De AK, Roach SE: Detection of the soluble heat shock protein 27 (hsp27) in human serum by an ELISA. *J Immunoassay Immunochem* 2004, 25(2):159–170. [PubMed: 15162919]
 49. Breiman L: Bagging Predictors. *Machine Learning* 1996, 24(2):123–140.
 50. Blackstone EH: Breaking down barriers: helpful breakthrough statistical methods you need to understand better. *J Thorac Cardiovasc Surg* 2001, 122(3):430–439. [PubMed: 11547291]

Figure 1.

Longitudinal mean change in extracellular HSP-70, HSP-27, IL-8 and IL-6 concentrations during 28 days of follow-up in surgical intensive care unit (SICU) patients by treatment group: **Panel 1A**, geometric mean serum HSP-70 (ng/mL), **Panel 1B**, mean serum HSP-27 (pg/mL), **Panel 1C**, mean serum IL-8 (pg/mL) and **Panel 1D**, mean serum IL-6 (pg/mL). Vertical bars=95% confidence intervals (CIs). No significant differences in mean serum HSP-70 (Panel A), mean serum HSP-27 (Panel B), mean serum IL-8 (Panel C) and mean IL-6 (Panel D) between the glutamine-PN (GLN-PN) and GLN-free, standard PN groups (STD-PN).

Figure 2.

Longitudinal mean change in extracellular HSP-70, HSP-27, IL-8 and IL-6 concentrations during 28 days of follow-up in surgical intensive care unit (SICU) patients by survival status:

Panel 2A, geometric mean serum HSP-70 (ng/mL), **Panel 2B**, mean serum HSP-27 (pg/mL), **Panel 2C**, mean serum IL-8 (pg/mL) and **Panel 2D**, mean serum IL-6 (pg/mL).

Vertical bars=95% confidence intervals (CIs). Patients that died had higher mean serum levels of HSP70 than patients who survived ($P = 0.14$, $P = 0.002$, $P < 0.001$, $P < 0.001$, $P < 0.001$ at 0, 3, 7, 14, 21, and 28 days of follow-up). There were no significant differences in HSP-27, IL-8, and IL-6 levels between 28-day survivors and non-survivors.

Figure 3.

Longitudinal mean change in extracellular HSP-70, HSP-27, IL-8 and IL-6 concentrations during 28 days of follow-up in surgical intensive care unit (SICU) patients by development of new hospital pneumonia: **Panel 3A**, geometric mean serum HSP-70 (ng/mL), **Panel 3B**, mean serum HSP-27 (pg/mL), **Panel 3C**, mean serum IL-8 (pg/mL) and **Panel 3D**, mean serum IL-6 (pg/mL). Vertical bars=95% confidence intervals (CIs).

Patients with an incident pneumonia diagnosis had higher mean serum levels of HSP-70 than patients without incident pneumonia ($P = 0.002$ for HSP-70 pooled across day of study and $P = 0.43$, $P = 0.36$, $P = 0.01$, $P < 0.001$, $P < 0.001$ and $P < 0.001$ at 0, 3, 7, 14, 21, and 28 days of follow-up, Panel 3A).

Patients with an incident pneumonia diagnosis had higher mean serum HSP-27 levels than patients without incident pneumonia ($P = 0.005$ for HSP-27 data pooled across day of study and $P = 0.16$ at baseline and $P = 0.08$, $P = 0.002$, $P = 0.005$, $P = 0.15$ and $P = 0.03$ at 3, 7, 14, 21, and 28 days of follow-up, respectively, Panel 3B).

Patients with an incident pneumonia diagnosis had higher mean IL-8 levels than patients without incident pneumonia ($P = 0.046$ for IL-8 data pooled across day of study and $P = 0.55$, $P = 0.32$, $P = 0.04$, $P = 0.07$, $P = 0.29$ and $P = 0.14$ at baseline and 3, 7, 14, 21, and 28 days of follow-up, respectively, Panel 3C).

Patients with an incident pneumonia diagnosis had higher mean IL-6 levels than patients without incident pneumonia ($P = 0.0067$ for IL-6 data pooled across day of study and $P = 0.77$, $P = 0.05$, $P = 0.10$, $P = 0.03$, $P = 0.02$ and $P = 0.15$ at baseline and 3, 7, 14, 21, and 28 days of follow-up, respectively, Panel 3D).

Figure 4. Longitudinal mean change in extracellular HSP-70, HSP-27, IL-8 and IL-6 concentrations during 28 days of follow-up in surgical intensive care unit (SICU) patients by development of new bloodstream infection (BSI): **Panel 4A**, geometric mean serum HSP-70 (ng/mL), **Panel 4B**, mean serum HSP-27 (pg/mL), **Panel 4C**, mean serum IL-8 (pg/mL) and **Panel 4D**, mean serum IL-6 (pg/mL). There were no significant differences in HSP-70, HSP-27, IL-8 and IL-6 levels between patients diagnosed with incident BSI and patients without incident BSI.

Figure 5.

The average rate of increase in serum HSP70 levels as serum IL-8 levels increased was similar for survivors and non-survivors ($P=0.97$, **Panel 5A**). Similarly the average rate of increase in serum concentrations of HSP70 as IL-8 increased was similar for SICU patients with and without incident pneumonia ($P=0.17$, **Panel 5B**). The slope or average rate of increase in serum HSP70 levels as IL-6 increased was different for survivors and non-survivors ($p=0.047$, **Panel 5C**). The slope or average rate of increase in serum levels of HSP70 as IL-6 levels increased was different for SICU patients with and without incident pneumonia ($p=0.054$, **Panel 5D**). Vertical bars=95% Cis

Table 1.

Univariable risk factors potentially associated with hospital mortality in SICU patients.

Risk Factor	Hospital mortality No./total (%)	Relative Risk	95% CI	P value
HSP70 at baseline, ng/mL				0.02
median 36	17/73, 23.3%	Reference		
< median 36	6/73, 8.2%	0.35	0.15, 0.84	
Rate of HSP70 decline/day				0.0005
median (slower decline)	19/73, 26.0%	Reference		
< median (faster decline)	4/75, 5.3%	0.20	0.07, 0.57	
HSP27 at baseline, pg/mL				0.82
median 1159	12/73, 16.4%	Reference		
< median 1159	11/73, 15.1%	0.92	0.43, 1.94	
IL-8 at baseline, ng/mL				0.82
median 46	12/74, 16.2%	Reference		
< median 46	11/74, 14.9%	0.92	0.43, 1.95	
IL-6 at baseline, ng/mL				0.82
median 118	12/74, 16.2%	Reference		
< median 118	11/74, 14.9%	0.92	0.43, 1.95	
Apache II at admission				0.14
median 23.0	15/75, 20.0%	Reference		
< median 23.0	8/73, 11.0%	0.55	0.25, 1.21	
On mechanical ventilation at entry				0.0047
Yes	21/97, 21.7%	Reference		
No	2/51, 3.9%	0.34	0.14, 0.82	

* The relative risk (RR) of hospital mortality per 10 year decrease in age (continuous covariate): RR = 0.67, P = 0.008).

Table 2.

Multivariable analysis of serum HSP70 concentration as a predictor of outcome in surgical ICU patients

Outcome	HSP70 (ng/mL)	Number,% with Outcome	Adjusted Relative Risk	95% CI	P Value	Area under ROC Curve	95% CI	Reliability (%) [†]
Hospital Mortality (n=148)	Slope <median (faster decline)	4/75, 5.3%	0.25	0.09, 0.68	0.001	0.81	0.72, 0.91	100%
	Slope median (slower decline)	19/73, 26.0%						
Pneumonia (n=148)	Slope <median	4/75, 5.3%	0.25	0.09, 0.70	0.008	0.80	0.69, 0.91	98%
	Slope median	18/73, 24.7%						
BSI (n=148)	Slope <median	12/75, 16.0%	0.78	0.39, 1.57	0.49	0.58	0.47, 0.70	3%
	Slope median	16/73, 21.9%						
Hospital mortality or Pneumonia (n=148)	Slope < median	6/75, 8.0%	0.24	0.11, 0.52	<0.001	0.86	0.79, 0.94	100%
	Slope median	30/73, 41.1%						

[†]Percentage of time HSP70 appears in 1000 bootstrap models. Risk factors appearing in ≥50% of models are reliable. All models adjusted for age at enrollment, mechanical ventilation at entry and the linear rate of decline of log HSP70. Rates of log serum HSP70 concentration decline over 28 days of follow-up were obtained by using a mixed-effects model specifying that HSP70 measurements follow a linear regression over time, with random intercept and slope for each patient. The calculated risk of hospital mortality is as follows: $e^w/(1+e^w)$, where $w = -3.4229 + (1.3804 \times X_1) + (1.4458 \times X_2) + 0.036X_3$ and X_1 is HSP70, X_2 is mechanical ventilation status at entry and X_3 is age (years) at enrollment. The estimated intercept $\pm$ standard error and the regression coefficient (β) $\pm$ standard error for each predictor in the adjusted model: -3.4229 ± 0.9794 and 1.3804 ± 0.5042 (estimated β for slower HSP70 decline), 1.4458 ± 0.7021 (estimated β when on mechanical ventilation at entry) and 0.0360 ± 0.0139 (estimated β for enrollment age, per 1 year increase). The estimated risk of hospital mortality is 83% for a SICU patient who at enrollment is 60 years of age, on a ventilator at entry and has a slower rate of linear decline in HSP70 (below the median rate of decline). Hospital Mortality: The area under the ROC curve is 0.85 (95% CI: 0.76, 0.94; see Figure) for the rate of linear decline per day in log HSP70 (the slope as a continuous covariate).