

Increased extracellular heat shock protein 90 α in severe sepsis and SIRS associated with multiple organ failure and related to acute inflammatory-metabolic stress response in children

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Abstract

Mammalian heat-shock-protein (HSP) 90 α rapidly responds to environmental insults. We examined the hypothesis that not only serum HSP72 but also HSP90 α is increased in the systemic inflammatory response syndrome (SIRS), severe-sepsis (SS), and/or sepsis (S) compared to healthy children (H); we assessed HSP90 α relation to (a) multiple organ system failure (MOSF) and (b) inflammatory-metabolic response and severity of illness.

A total of 65 children with S, SS, or SIRS and 25 H were included. ELISA was used to evaluate extracellular HSP90 α and HSP72, chemiluminescence interleukins (ILs), flow-cytometry neutrophil-CD64 (nCD64)-expression.

HSP90 α , along with HSP72, were dramatically increased among MOSF patients. Patients in septic groups and SIRS had elevated HSP90 α compared to H ($P < 0.01$). HSP90 α was independently related to predicted death rate and severity of illness; positively to HSP72, nCD64, ILs, length of stay, days on ventilator, and fever; negatively to HDL and LDL ($P < 0.05$). The HSP72 was increased in SS/S and related negatively to HDL and LDL ($P < 0.05$).

Serum HSP90 α is markedly elevated in children with severe sepsis and is associated with MOSF. Better than the HSP72, also increased in SS, SIRS, and MOSF, HSP90 α is related to the inflammatory stress, fever, outcome endpoints, and predicted mortality and inversely related to the low-LDL/low-HDL stress metabolic pattern.

Abbreviations: HDL = high-density-lipoprotein, HSP = heat shock protein, ILs = interleukins, LDL = low-density lipoprotein, LOMV = length of mechanical ventilation, LOS = length of stay, MOSF = multiple organ system failure, PELOD = pediatric logistic organ dysfunction, PRISM = pediatric risk of mortality, SIRS = the systemic inflammatory response syndrome, TISS = the Therapeutic Intervention Scoring System, TLR4 = toll-like-receptor-4.

Keywords: children, fever, heat-shock-protein, metabolism, organ failure, sepsis, SIRS

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1. Introduction

Severe infections kill >4.5 million children every year, representing a major public health problem. More patients with sepsis (S) (5.6%) and sepsis/septic shock (SS) (17.0%) die compared with mortality of all pediatric intensive care unit (PICU) (3.0%) admissions. The noninfectious systemic inflammatory response syndrome (SIRS) is also a major cause of disability or death. Although clinical adherence to the Pediatric Guidelines for septic shock has been shown to influence outcome, no targeted treatment to sepsis and/or SIRS underlying excessive immune response has been found so far.

An overwhelming immune response in infectious (S, SS) or noninfectious SIRS, as mediated by the uncontrolled release of proinflammatory mediators, can lead to shock, multiple organ system failure (MOSF), and even death. Elevated neutrophil CD64 (nCD64) expression was reported in adult patients and in children with sepsis.^[1] The molecules, however, that promptly activate immune competent cells through the LPS-TLR4-CD14-dependent pathway, acting as "danger signals," are extracellular heat shock proteins (HSPs). Extracellular HSPs released from stressed cells serve to signal an impending "danger signal" to neighboring and immune cells (danger hypothesis), presumably promoting immune responses and improving host defense.^[2]

It has been previously shown that an intense stressor exposure may increase the 70-kDa family of HSP (inducible HSP72), which

acts as a danger “alarm” to potentiate the nitric oxide (NO) stress-response and engender recovery from bacterial inflammation^[3] or heat stress.^[4] Contrasting to animal studies showing an HSP72 protective effect in experimental sepsis, human in vivo or in vitro studies are not conclusive, showing a possible relation to either protection or mortality and morbidity.^[5] Although increased circulation of stress proteins signals danger to inflammatory cells and aid in immune surveillance, they have also been linked to a deleterious role in some diseases.^[6] Thus, increased extracellular HSP72 levels were detected in the plasma of children admitted to the PICU with septic shock and correlated with worse outcome.^[7] Extracellular HSP72 has previously been shown to be elevated not only in patients with septic shock, but also following cardio-pulmonary bypass or in children with acute lung injury.^[8]

Another major molecular chaperone, 90-kDa family of HSP (inducible HSP90 α), has been shown to interact with about 200 client proteins, mainly involved in transcription regulation and signaling-transduction pathways of glucocorticoid receptors, ribonucleoproteins, chromatin remodeling factors, transcription factors, and protein kinases.^[9] The guarding role of this abundant “chaperone,” the intracellular HSP90 α , is to launch a rapid response to various insults such as heat, hypoxia, reactive oxygen species, and injury-released growth factors.^[10–11] Thus, a well-characterized function of secreted HSP90 α is to boost cell motility, facilitating tissue healing.^[12] Under hypoxia, HSP90 α is secreted into extracellular space, binding and signaling through the low-density-lipoprotein (LDL)-receptor-related protein-1 to promote cell motility, finally leading to repair of damaged tissues.^[13] In addition, the secretion of HSP90 α , but not HSP90 β , is increased in activated endothelial cells, promoting their angiogenic activities with the induction of matrix proteins.^[14]

Following the contrasting results shown by HSP72 human studies,^[5] it has been recently shown that the activation of the HSP90 α /Akt pathway induces caspase-3 activation, provoking thereby apoptosis in septic mice.^[15] In addition, inhibition of HSP90 α prevented ischemia-reperfusion injury in the kidney donor and/or organ,^[16] and suggested to open the route towards novel treatment modalities of severe sepsis associated acute lung injury.^[11] Robust correlations, however, between extracellular HSP90 α levels with clinically relevant outcome variables during the acute phase of SS or SIRS or with pro- or anti-inflammatory interleukins (IL), nCD64 or metabolic derangements are missing. Similarly, no studies in children have been conducted comparing HSP90 α with HSP72, a HSP90 α -machinery complement, in SS and/or SIRS, or relating their levels to organ failures, severity of illness and predicted or measured outcome endpoints.^[17] We hypothesized that increased HSP90 α levels could be detected in the serum of critically ill children, especially those with severe sepsis and/or septic shock and in SIRS compared to healthy controls (H). We also hypothesized that the abundant intracellular chaperone HSP90 α —when released extracellularly as a chaperokine—might be better than the HSP72’s correlated with MOSF, early inflammatory and metabolic acute stress, severity of illness, and outcome endpoints.

2. Materials and methods

2.1. Setting and research ethics

This prospective observational study was conducted in the PICU of the University Hospital, Heraklion, University of Crete, between January 2010 and September 2014. The study was

approved by the Ethical Committee of the University Hospital, Heraklion, Crete, Greece. Written informed consent was obtained from patients’ guardians before inclusion in this study. All data were anonymous.

2.2. Study Patients

Sixty-five critically ill children, consecutively admitted to the PICU, with noninfectious (SIRS) (n=23) or infectious SIRS [S (n=20), and SS (n=22)] were enrolled in the study. Twenty-five healthy children undergoing screening tests for minor elective surgery were consisted in the control group. Allocation into groups was carried out within 6 hours of admission by the attending physician, who was unaware of the results of laboratory assays, until at least 20 patients were included in each group. Fifteen of the severe sepsis group fulfilled the criteria of septic shock; all septic shock and severe sepsis patients had infection, SIRS, and at least 2 organ failures, thereby representing a homogenous group (SS). Patients with immunodeficiency, autoimmunity, malignancies, and receiving steroids or with metabolic or chronic diseases were excluded (criteria predefined). Enrolled SIRS patients had trauma without infection (fractures, liver, spleen, or lung contusions). None of the patients received parenteral nutrition. After initial blood studies, patients were put on enteral nutrition according to our PICU protocol. The Pediatric Logistic Organ Dysfunction Score (PELOD),^[18] the simplified Therapeutic Intervention Scoring System (TISS),^[19] and the Pediatric Risk of Mortality Score (PRISM)^[20] were the recorded markers of severity of disease on the day of blood sampling. The predicted death rate was calculated after PRISM. Length of stay (LOS), days on mechanical ventilation (DOMV), and PICU-mortality were the outcome endpoints recorded. Sepsis, severe sepsis, septic shock, and SIRS were defined according to the *International Pediatric Sepsis Consensus Conference* definitions. The MOSF was defined using the criteria by Wilkinson (presence of at least 2 organ system failures).^[21] According to the exclusion criteria, patients with cancer, immune-deficiencies (immunosuppressive agents but not stress-dose hydrocortisone), and metabolic diseases or endocrinopathies (diabetes, hypercholesterolemia) were not included.

2.3. Laboratory evaluations

Admission blood and urine cultures, pharyngeal and deep tissue swabs, and bronchoalveolar lavage (BAL) were routinely taken in all patients to the PICU, despite the fact that most of the critically ill patients had already received antibiotics prior to their admission to the intensive care unit.

Blood was drawn for routine exams at the time of admission. Venous blood was processed to measure white blood count (WBC), procalcitonin (PCT), and C-reactive protein (CRP) levels. Samples for enzyme-linked immunosorbent assay (ELISA) or chemiluminescence were collected in tubes and were centrifuged immediately at 4000 rpm 10 minutes to separate serum from the cellular components. Samples were stored at -80°C until later analysis. Investigators were blinded to the patients’ group assignment and available clinical data until after the analysis.

2.4. Elisa immunoassay

Extracellular protein levels of HSP90 α and HSP72 were determined by ELISA commercial kits obtained from Enzo Life Sciences (Lausanne, Switzerland) according to manufacturer’s

instructions. Samples underwent a 1:25 dilution for HSP90 α and 1:1 for HSP72 and then were pipetted into the wells precoated with specific mouse-monoclonal antibody. Following incubation and washing, a horseradish peroxidase antibody specific for HSP90 α was added to the wells, whereas this phase was performed in 2 steps for the HSP72. Following washing, the substrate solution was added and color developed. The development of color was stopped and the optical density of each well was measured in an automated microplate reader set at 450 nm. All measurements were performed in duplicate. The sensitivities of the assays were 50 pg/mL for HSP90 α and 90 pg/mL for Hsp72.

2.5. Chemiluminescence

Tumor necrosis factor alpha (TNF- α), IL-6, IL-10, and IL-8 were measured using a commercially available chemiluminescence kit (IMMULITE 100 Siemens Medical Solutions Diagnostics Limited, Surrey, UK). The sensitivities of the assays were <2 pg/mL for IL-6, <1 pg/mL for IL-10, 2 pg/mL for IL-8, and 4 pg/mL for TNF- α . All samples were processed in duplicate with mean value used if the coefficient of variance was <20%.

2.6. Flow cytometry

Whole blood, EDTA-anticoagulated samples were immediately transported to the laboratory for flow cytometric CD64 analysis. In brief, 100 μ l of the whole blood sample were incubated with phycoerythrin (PE)-conjugated CD64 (Clone 22) for 20 minutes at room temperature, in the dark. PE-mouse IgG was used as the isotype control (antibodies from IMMUNOTECH-BECKMAN COULTER, Marseille, France). Labeled cells were thoroughly washed with PBS+2 % FCS+ sodium azide. Following lysis in immunoprep reagent system (BECKMAN COULTER, Mervue, Galway, Ireland), the cells were analyzed on an Epics-Coulter cytometer. They were initially gated according to their morphology (forward- and side-scatter characteristics) so that >30,000 cells were acquired in each sample. Results are presented as the percentage (%) of positive staining cells. Analyses were performed in monocyte, lymphocyte, and neutrophil populations.

2.7. Routine laboratory measurements

CRP assay was performed on the immunonephelometer Dade Behring BN II (Dade Behring Diagnostics Inc., Somerville, NJ). Abnormal were considered CRP concentrations >0.8 mg/dL. Procalcitonin (PCT) was measured by an immunoluminometric assay (Lumitest-PCT, Brahms-Diagnostica, Berlin, Germany) with a detection threshold at 0.1 ng/mL. According to the laboratory cut-off values, PCT levels >0.5 ng/mL were considered abnormal. Plasma concentrations of glucose, triglycerides (TG), total cholesterol (TC), high-density lipoproteins (HDL) or LDL, and hematological markers were measured by routine laboratory methods.

2.8. Statistical analysis

The 1-sample Kolmogorov–Smirnov test was used in order to determine the data distribution from measured variables. Data are expressed as median values with range for continuous parameters and as frequencies for categorical variables. The Kruskal–Wallis and the Mann–Whitney tests were used to perform comparisons among groups, as appropriate. We ran

multiple comparison analyses using *post hoc* Dunn's pairwise tests with Bonferroni corrections for the Kruskal–Wallis H test; the χ^2 test statistic was adopted to calculate the effect size ($H^2 = \chi^2/(n - 1)$) in order to avoid the inflation of overall type I error. Respectively, the Mann–Whitney U -values with z -scores were used to calculate the effect size ($r = z/\sqrt{n}$) in order to measure the size of differences. Between-group comparisons were conducted using the χ^2 test for categorical parameters and Spearman rank correlation coefficient for correlation between 2 continuous variables. Although the correlation coefficients usually serve as the effect size index, to further estimate the effect size, we converted the correlations to Fisher's z scales to calculate the sizes of associations. Finally, a multivariate linear regression analysis (stepwise method) was adopted to examine whether HSP90 α was an independent predictor of outcome endpoints. A 2-sided significance level of 0.05 was used for statistical inference. All statistical analyses were performed using the IBM SPSS Statistics (version 22.0; Chicago, IL).

3. Results

There were no statistical differences regarding gender in the studied groups. Septic, but not severely septic, patients were younger compared to healthy and SIRS groups. Two patients in the SIRS group—both having suffered multiple injuries from motor vehicle accidents (the patients with the highest PRISM and TISS)—and 2 patients with septic shock (multiple organ systems failure) died. Patients with severe sepsis had higher first-day temperature, severity scores, organ failures, and longer LOS, and DOMV compared to the sepsis or SIRS groups (Table 1). The calculated effect size was larger for MOSF and fever, whereas it was better for predicted than actual mortality. Would we had applied the recently updated SEPSIS-3 definition^[22] for adults in our pediatric population, we would have found the same statistical differences among groups. The only difference noted by running such a pilot study has been that SEPSIS-3 septic shock should have replaced SS group, and SEPSIS-3 sepsis would correspond to our S group (except 1 patient moving from the SS to S group).

In the 2 septic groups, pathogens isolated in the PICU were as follows: blood: *Streptococcus pneumoniae*, *Acinetobacter baumannii*, *Enterococcus faecalis*, *Escherichia coli*, (n=1 each), *Neisseria meningitidis* and *Candida* species (n=2 each) and *Staphylococcus haemolyticus* (n=4); BAL: *Pseudomonas aeruginosa* (n=5), *Serratia marcescens*, *Stenotrophomonas maltophilia*, *Neisseria*, *Mycoplasma pneumoniae* (n=1 each); urine: *Pseudomonas aeruginosa* (n=2), *Escherichia coli* (n=1); Central nervous system: *N meningitidis* (n=1). Patients without isolates had received antibiotics before PICU admission in another hospital (cultures not sent or results unavailable). No pathogens were isolated from the blood and samples from other sites in SIRS patients.

Patients in S, SS, and SIRS groups had elevated HSP90 α compared to H (Fig. 1A). Septic groups, especially SS, had also increased HSP72 compared to H and/or SIRS (Fig. 1B). The HSP90 α elevation paralleled: an increase of HSP72, IL-6, IL-8, and IL-10 levels in septic groups compared to H; elevated HSP72, IL6, and TNF- α in SS compared to SIRS; elevated IL-6 and IL-8 in SIRS compared to H (Table 2). The calculated effect size was better for HSP90 α and IL-6.

HSP90 α , along with HSP72, were dramatically increased among MOSF patients (Fig. 2A and B). The HSP90 α and HSP72 increases in the MOSF group paralleled: (a) significant increases

Table 1

Baseline characteristics on study enrollment.

	Healthy control	SIRS	Sepsis	Severe sepsis	Chi-square (<i>df</i>) [*]	H ² effect size ^{**}	P ^{***}
Subjects, n	25	23	20	22			
Gender male, n, %	14 (66%)	13 (72%)	10 (62%)	9 (60%)	0.64 (3)	0.01	0.8
Age, y	9 (2–15.5) [¶]	8.5 (2–18)	4.25 (1–18) ^{,¶}	6 (1–18)	8.8 (3)	0.13	0.029
Mortality, n	NA	2	0	2	4.5 (2)	0.04	0.5
Predicted death, %	NA	7.6 (1.3–77)	7.6 (1.3–34)	13.4 (1.3–60)	4.6 (2)	0.10	0.1
MOSF, n	NA	9	0	22	41.3 (2)	0.75	0.001
PRISM II	NA	11 (6–29)	11 (2–20)	15 (6–26)	4.6 (2)	0.12	0.12
PELOD	NA	3 (1–21) [‡]	2 (1–13) [§]	13 (5–32) ^{‡,§}	12.9 (2)	0.32	0.001
TISS	NA	38.5 (25–57)	36.5 (12–47) [§]	42 (32–59) [§]	4.7 (2)	0.12	0.14
TEMP MAX, °C	NA	36 (35–39)	38.9 (38–40) ^{‡,}	39.5 (38–41) ^{‡,}	21.6 (2)	0.54	0.001
LOS, days	NA	6.5 (3–18) [‡]	10.5 (2–98)	16 (3–235) [‡]	7.0 (2)	0.18	0.03
DOMV, days	NA	4.5 (1–14) [‡]	6 (1–95)	11 (1–235) [‡]	6.1 (2)	0.15	0.05

LOMV=duration of mechanical ventilation, LOS=length of stay, MOSF=multiple organ system failure, PELOD=pediatric logistic organ dysfunction, PRISM=pediatric risk of mortality, TEMP MAX=temperature maximum first day, TISS=the Therapeutic Intervention Scoring System.

Independent Samples Kruskal–Wallis Test; Data given as median (range).

* Test statistic χ^2 .

** Effect size ($\chi^2/[n-1]$).

*** Asymptomatic Significance (2-sided test).

† SS vs. H.

‡ SS vs. SIRS.

§ SS vs. S.

|| S vs. SIRS.

¶ S vs. SIRS.

of the inflammatory biomarkers nCD64, IL-6, CRP, and PCT; (b) significant decreases of the nutritional indices cholesterol, HDL, and LDL, but not glucose (Table 3). The effect sizes (*r*) were larger for HSP90 α , CRP, PCT, IL-6, LDL, HSP72, and nCD64.

HSP90 α related positively to IL-8, IL-10, IL-6, and CRP (Fig. 3A–D). HSP90 α was also related to ventilator days, LOS, organ dysfunction (PELOD), PRISM-predicted death rate (Fig. 3E–H), PCT ($r_s=0.51$, $P<0.001$), TISS ($r_s=0.44$, $P<0.02$), and first-day maximum temperature ($r_s=0.38$, $P<0.045$). TNF ($r_s=0.47$, $P<0.03$), nCD64 ($r_s=0.64$, $P<0.001$), CRP ($r_s=0.67$, $P<0.001$), and PCT ($r_s=0.72$, $P<0.03$), but not HSP72, IL-8, or IL-10, were also related to the first-day maximum temperature. In all cases, renal clearance was adjusted for in the associations between HSP and illness severity/mortality. The calculated mostly large effect size Fisher's Z_r scales were for Fig. 3A=0.74, b=0.87, c=0.56, d=0.52, e=0.45, f=0.69, g=0.91, h=0.35. Despite a wide scattering,

HSP90 α exhibited a positive relation to HSP72, nCD64 and stress-related metabolic molecules glucose and triglycerides (Fig. 4A–D). Even more importantly, HSP90 α and HSP72 related negatively to both lipoproteins (Fig. 4E–H). The calculated effect size by Fisher's Z_r scales was rather medium in this case: Fig. 4A=0.38, b=0.51, c=0.55, d=0.43, e=0.52, f=0.39, g=0.42, h=0.30.

Despite the small mortality rate, not only HSP90 α (682 [520–1716] vs. 121 [18–625] ng/mL, $P<0.0001$) but also HSP72 (0.41 [0.24–1.4] vs. 0.13 [0–1] ng/mL, $P<0.015$) were increased among nonsurvivors. The small mortality rate in this study could not allow performing a binary logistic analysis. Instead, as predicted mortality was related to mortality ($r=0.53$, $P<0.01$) and the 2 variables did not differ ($P<0.06$), multiple linear regression analysis was performed (stepwise method) to examine if any of the HSPs, ILs, inflammatory biomarkers (CD64, CRP, PCT), and nutritional indices could independently predict

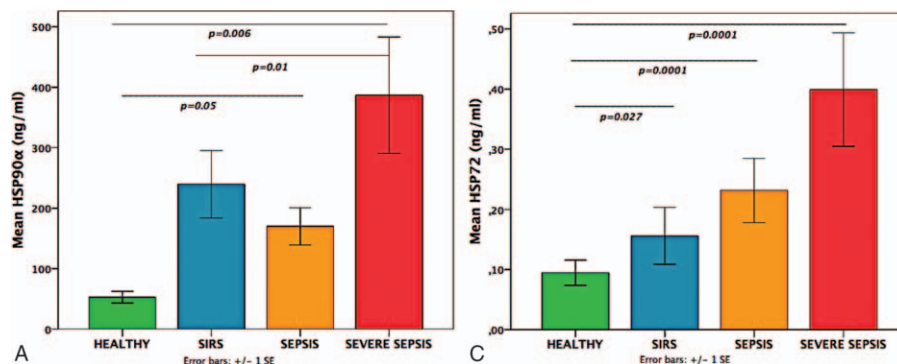


Figure 1. (A) Patients with severe sepsis, sepsis, or SIRS had increased extracellular HSP90 α in comparison to healthy controls; (B) patients with severe sepsis or sepsis had increased extracellular HSP72 in comparison to healthy controls and/or SIRS (Mann–Whitney tests). HSP=heat shock protein, SIRS=systemic inflammatory response syndrome.

Table 2
First-day serum concentrations and group differences.

	Healthy control	SIRS	Sepsis	Severe sepsis	Chi-square (df)*	H ² effect size**	P***
HSP90α, ng/mL	35.5 (18–148) ^{†,¶,♯}	133 (19–773) [#]	156 (21–520) [¶]	298 (47–1716) [†]	21.5 (3)	0.31	0.0001
HSP72, ng/mL	0.11 (0.01–0.22) [†]	0.1 (0.03–0.66)	0.25 (0.07–0.58)	0.19 (0.09–1.39) [†]	10.2 (3)	0.15	0.017
IL-8, ng/mL	6.4 (4–32) [†]	25 (7.4–95.8)	22 (7–861)	84 (6–7100) [†]	9.8 (3)	0.14	0.02
IL-10, ng/mL	4.6 (4–32) ^{†,¶}	11 (7–177)	17 (7–1100) [¶]	38 (4–876) [†]	12.1 (3)	0.18	0.07
TNF-α, pg/mL	13 (9–65)	12 (9–15) [‡]	15 (7–66)	22 (11–95) [‡]	8.2 (3)	0.12	0.043
IL-6, pg/mL	1 (1–11) ^{†,¶,♯}	20 (2–77) ^{‡,♯}	15 (3.5–1100) [¶]	104 (22–1100) ^{†,‡}	16.2 (3)	0.23	0.001

HSP = heat shock protein, IL = interleukin, SIRS = systemic inflammatory response syndrome, TNF-α = tumor necrosis factor alpha.

Independent Samples Kruskal–Wallis test; data given as median (range).

* Test statistic χ^2 .

** Effect size ($\chi^2/[n-1]$).

*** Asymptomatic Significance (2-sided test).

[†] SS vs. H.

[‡] SS vs. SIRS.

[¶] S vs. SIRS.

[#] SIRS vs. H.

outcome (predicted mortality). Only HSP90α was an independent predictor of predicted mortality (Beta 0.55, 95% CI 0.02–0.04, $P < 0.001$). HSP90α was also independently associated with MOSF (PELOD) (Beta 0.51, 95% CI 0.01–0.02, $P < 0.001$) as were TNF (Beta 0.45, 95% CI 0.9–0.3, $P < 0.002$) and IL-10 (Beta –0.31, 95% CI –0.02– (–0.002), $P < 0.02$). Finally, HSP90α was independently associated with the therapeutic intervention score (TISS) (Beta 0.38, 95% CI 0.005–0.02, $P < 0.002$) as was IL-10 (Beta –0.37, 95% CI –0.02– (–0.005), $P < 0.002$).

4. Discussion

We showed that serum concentrations of HSP90α are markedly elevated in children with severe sepsis and to a lesser degree in critically ill children with sepsis or SIRS. We also showed that HSP90α is dramatically increased among patients with organ failures, an increase better associated with early inflammatory-metabolic stress than HSP72. However, a parallel increase of extracellular HSP72 in patients with sepsis or MOSF was also shown. Finally, we showed that both HSPs are inversely related to the low-LDL/low-HDL stress metabolic pattern, revealing a possible role for the heat shock response in the acute metabolic derangement in sepsis and SIRS. Not irrelevant to early HSP90α-response to stress severity and MOSF-association, we showed

that extracellular HSP90α was independently related to PRISM-predicted death rate and the severity of organ dysfunction. In addition, HSP90α was related to fever, nCD64, IL-6, IL-8, IL-10, acute phase proteins, and length of stay or mechanical ventilation significantly more than extracellular HSP72.

The “danger hypothesis” suggests the release of stress proteins from severely stressed or damaged cells which serve as a danger signal to neighboring cells.^[23] HSP90α membrane translocation and cell secretion is through exosome protein secretory pathway^[24] or in a specific and inhibitible manner through the endo-lysosomal pathway from viable cells.^[25] Then, immediately after complex DNA damage the threonine 7 residue of HSP90α is phosphorylated^[26] and accumulated at sites of DNA double-strand breaks forming repair foci.^[24] An important finding of this study is a trend for an escalating response to stress (assuming DNA damage) of not only HSP90α, but also HSP72 in the SIRS and in the 2 septic groups compared to healthy children. In accordance to our findings of increased HSP72 serum levels associated with predicted death rate in children with SS and SIRS, increased extracellular HSP72 levels with septic shock have been previously correlated with mortality in adults^[27] and in children.^[7] Verifying its alarming role, circulating HSP72 has also been associated with organ dysfunction or complications in coronary artery disease,^[28] liver disease, vaso-occlusive sickle cell disease-crisis, and in pre-eclampsia.^[29] Extending these

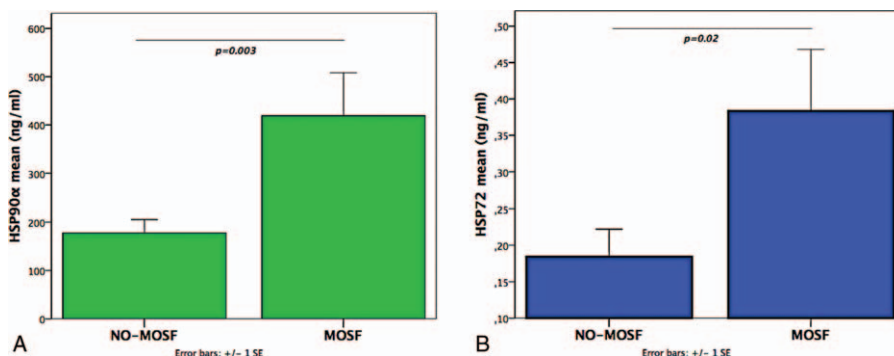


Figure 2. Patients with MOSF had increased. (A) Extracellular HSP90α and (B) extracellular HSP72 compared to non-MOSF patients (Mann–Whitney test). HSP = heat shock protein, MOSF = multiple organ system failure.

Table 3

Acute stress (first 24 h) levels of HSPs, inflammatory biomarkers, and nutritional indices in mechanically ventilated children with sepsis or SIRS classified according to their MOSF status.

	Non-MOSF	MOSF	Mann-Whitney <i>U</i>	Z score	Effect size (<i>r</i>) [*]	<i>P</i> ^{**}
Number, n	34	31				
HSP90α, ng/mL	141 (19–520)	340 (47–1716)	133	−2.93	−0.42	0.003
HSP72, ng/mL	0.13 (0.00–0.66)	0.26 (0.01–1.39)	160	−2.36	−0.34	0.018
ICD64, %	0.3 (0–3.8)	0.5 (0.1–2.9)	140	−0.99	−0.14	0.33
mCD64, %	87 (0–97)	91 (42–99)	133	−1.18	−0.17	0.25
nCD64, %	25.7 (0.5–99)	95 (3–100)	95	−2.32	−0.33	0.02
CRP, mg/dL	4.2 (0.3–25)	13 (0.3–41)	130	−2.99	−0.43	0.003
PCT, ng/mL	0.6 (0.3–12)	11 (0.3–12)	102	−3.34	−0.48	0.001
WBC, K/μL	12 (2–40)	11 (1.5–47)	224	−0.98	−0.14	0.33
IL-8, ng/mL	21 (7–861)	66 (6–7100)	55	−1.68	−0.24	0.99
IL-10, ng/mL	9 (4–1100)	38 (4–876)	60	−1.75	−0.25	0.08
TNF-α, pg/mL	12 (7–66)	17 (10–95)	64	−1.57	−0.23	0.12
IL-6, pg/mL	17 (2–1100)	91 (22–1100)	18	−2.63	−0.38	0.006
Glu, mg/dL	116 (31–332)	126 (77–265)	219	−1.24	−0.18	0.2
TGL, mg/dL	89 (27–326)	104 (34–591)	238	−0.68	−0.10	0.5
TC, mg/dL	126 (55–277)	100 (45–150)	170	−2.13	−0.31	0.03
HDL, mg/dL	40 (9–62)	24 (9–62)	167	−2.10	−0.30	0.04
LDL, mg/dL	68 (15–209)	40 (13–86)	125	−2.66	−0.38	0.008

CRP = C-reacting protein, HDL = high-density lipoproteins, HSP = heat shock protein, IL = interleukin, ICD64 = lymphocyte CD64, LDL = low-density lipoproteins, mCD64 = monocyte CD64, MOSF = multiple organ system failure, nCD64 = neutrophil CD64, PCT = procalcitonin, SIRS = systemic inflammatory response syndrome, TC = total cholesterol, TGL = triglycerides, TNF-α = tumor necrosis factor alpha, Glu, glucose, WBC = white blood cells.

Independent Samples Mann-Whitney *U*; Data given as median (range).

* Effect size ($r = z/\sqrt{n}$).

** Asymptomatic significance (2-sided test).

publications, we have now shown that higher extracellular HSP72 levels correlate with MOSF in critically ill children with SIRS and/or sepsis.

There are limited data describing extracellular HSP90α levels in adults, children, or neonates with severe sepsis. We have

recently shown that extracellular HSP90α levels are increased in adult SS and SIRS and that they are related to HSP72, interleukins, and cortisol.^[30] We are now extending this observation by revealing increased HSP90α in critically ill children, especially those with severe sepsis. Furthermore, we

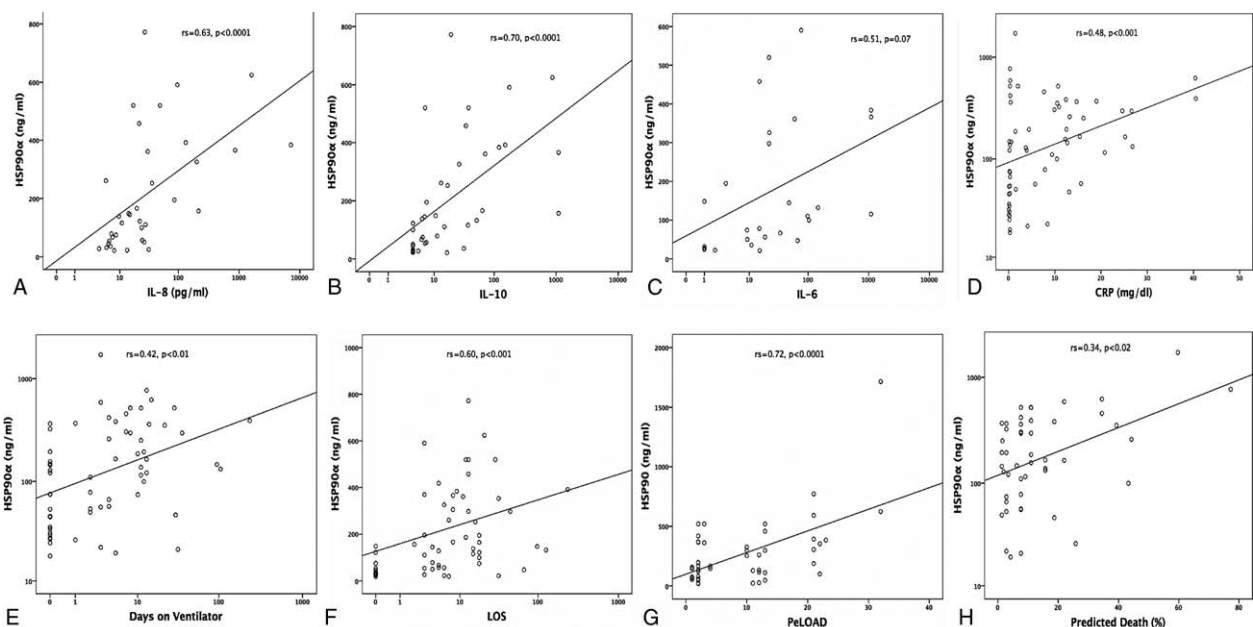


Figure 3. Positive correlations of HSP90α serum concentrations with serum levels of: (A–D) Interleukins and CRP; (E–H) days on ventilator, length of stay (LOS), organ dysfunction (PELOD), and PRISM-predicted death rate as indicated. Spearman rank correlation test, correlation coefficient *r*, and *P* values are given. CRP = C-reacting protein, HSP = heat shock protein, IL = interleukin, LOS = length of stay, PELOD = Pediatric Logistic Organ Dysfunction Score, PRISM = Pediatric Risk of Mortality Score.

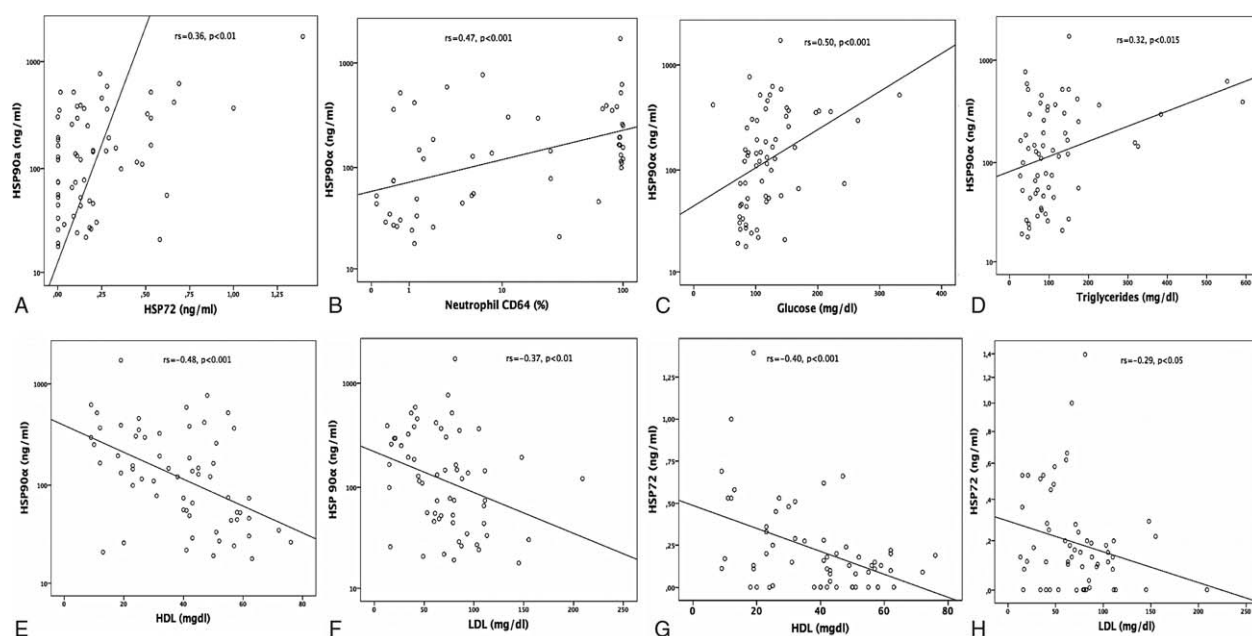


Figure 4. Positive correlations of HSP90α serum concentrations with: (A–D) Serum levels of HSP72, nCD64 expression, glucose and triglycerides; (E–H) negative correlations of HSP90α and HSP72 serum concentrations with LDL and HDL as indicated. Spearman rank correlation test, correlation coefficient r , and P values are given. HDL=high-density lipoproteins, HSP=heat shock protein, LDL=low-density lipoproteins, nCD64=neutrophil CD64.

found a wide HSP90α correlation to the nCD64, IL-6, IL-8, IL-10, severity of illness, LOS, days on ventilator and predicted death rate. These findings might simply indicate a proportionally greater level of physiologic stress associated with SS compared to SIRS. Thus, extracellular HSP90α, acting as a “chaperokine,” could have potentiated an already active systemic inflammatory response in SS. Our finding that HSP90α is inversely related to an early septic metabolic pattern indicates that HSP90α may induce a strong acute phase nCD64—interleukin inflammatory response resulting in an unbalanced metabolic status. We have previously shown that an early-onset sepsis-related stress-metabolic pattern is characterized by an acute increased [nCD64, glucose, triglyceride]—decreased [total-cholesterol, HDL, LDL] biomarker pattern, contrasting the moderate combination of noninfectious SIRS in which glucose increase is combined with a nonsignificant cholesterol-lipoprotein decrease.^[31] This is the first time that higher extracellular HSP90α levels are shown to be independently associated with mortality (predicted), MOSF, severity of illness scores, and therapeutic interventions (TISS). Plasma HSP90α has recently been reported to be a potential predictor for poor outcome in septic adults.^[32] Also, in a murine model of sepsis, HSP90α inhibitors improved pulmonary and circulatory function and attenuated systemic and pulmonary inflammation.^[11]

Our data, revealing an alarming increase of HSP90α among MOSF patients, assume that HSP90α may have a central clinical role to play in septic and/or SIRS patients. It has been previously proposed that MOSF in sepsis may be due to an early hyper-inflammatory “cytokine storm” response or to an early activation of innate immunity and suppression of adaptive immunity.^[33] Although most patients restore innate and adaptive immunity and survive the infectious or noninfectious stress, some patients enter a state of either profound immunosuppression or persistent activation of the innate immunity, with resultant secondary infections or intractable inflammation and multiple organ failure,

respectively.^[33] In our study, serum HSP90α differences suggest a double pathogenic mechanism not only in severe sepsis but also in noninfectious SIRS, roughly discriminating between healthy and critically ill, and between MOSF and non-MOSF patients. It has been suggested that dendritic and immune cell receptors capture HSP released from HSP-containing exosomes and release chemokines and interleukins.^[34] This may explain the strong HSP90α–IL relation shown in this study. The host innate immune response occurs through an NF- κ B-dependent pro-inflammatory gene expression via TLR4 and TLR2, similar to an LPS-mediated signal transduction.^[35] Similarly, the inducible isoform HSP90α mediates LPS-signaling as a part of the LPS receptor cluster, involving reactive oxygen species, cell apoptosis,^[36] and the regulation of IL-1 gene expression.^[37]

In our study, extracellular HSP90α was significantly correlated with IL-8, previously shown to be associated with LPS-induced caspase-3 activation and apoptosis.^[38] Furthermore, intracellular HSP72 and HSP90α exhaustion, which occurs as sepsis progresses,^[39] results in a progressive decrease of HLA-DR on CD14+ monocytes in severe sepsis.^[39] Importantly, intracellular HSP90α expression was recently shown to improve cellular resistance to heat-shock and apoptosis, a positive effect attenuated by HSP90α pre-stress inhibition.^[40] Similarly, HSP72 mRNA and intracellular HSP72 proteins were higher in sepsis, further induced by heat-shock and related to serum ILs.^[41] In our study, HSP90α, TNF, IL-6, and nCD64 were also correlated with fever. Hypoxia-inducible factor-1 alpha and HSP90α can orchestrate temperature-dependent gene transcription and involve in thermal adaptation.^[42]

Several limitations of our experimental approach should be mentioned. Given the number of strict exclusion criteria applied to assure homogeneity of our subcohorts and the rarity of nonimmune-compromised patients with severe sepsis, our sample size is relatively small, although it is larger compared to the sample size of similar studies.^[27,43] Accordingly, it is not possible

to establish the function of HSP90 α in circulation during disease in such a translational study. Subanalyses of interacting effects of specific therapeutic interventions on extracellular HSP90 α concentrations were not conducted due to the size of patients in our cohorts. However, by using adrenaline/noradrenaline as first choice inotropes/vasoacting agents, we did not have patients receiving dobutamine, recently shown to induce DNA-binding of HSF-1 and mRNA-expression of HSP90 α .^[44] During the study period, none of our patients was receiving thiopental that specifically and differentially induces a heat shock response, mediating thereby cytoprotection via the expression of HSP72 in human T-lymphocytes.^[45] Another limitation of the study is that we did not determine extracellular HSP concentrations beyond the first day of hospitalization in PICU. The possible fluctuations of extracellular and intracellular HSP90 α and HSP72 concentrations over time are currently under examination by our group.

Novel immune-metabolic and neurophysiological mechanisms underlie altered cellular bioenergetics and organ failure.^[46] In particular, the secretion and extracellular action of HSP90 α has been proposed to be a critical step downstream of the hypoxia-inducible factor-1 α pathway, leading to enhanced cell migration and wound healing.^[47] Our study findings, showing an early increase of extracellular HSP90 α in acute severe stress and its relation not only to the protective nCD64, IL-10 but also to the inflammatory IL-6, IL-8, PCT, CRP, and PELOD, and to the suppressed LDL and HDL, might imply HSP90 α as a contributing factor not only in the remodeling of damaged organ tissues but also in provoking apoptosis in severe sepsis.^[15] This is in accordance to experimental studies showing that HSP90 inhibitors improve survival and ameliorate inflammation, shock, and organ dysfunction.^[48] It has been already reported that targeting HSP90 α provides protection against intestinal inflammation and leakage and has been proposed to be a useful strategy to ameliorate intestinal failure in polymicrobial sepsis.^[49] Risk models for all-ages' critical illness, based on multimarker genomics, lipidomics and metabolomics,^[50] could possibly include HSP72 and HSP90 α , thus making HSP90 α a promising tool in the future improvement of the therapeutic approach of severe sepsis and MOSF.

5. Conclusions

In the present study, we report for the first time that the extracellular HSP90 α is significantly elevated in the critically ill with SIRS, especially in pediatric patients with severe sepsis. More importantly, we showed the extracellular "chaperokine" HSP90 α is associated with MOSF, inflammatory stress, severity of illness, organ dysfunction, length of stay, days on ventilator, and predicted mortality. The HSP90 α and HSP72 inverse relations to the low-LDL/low-HDL early septic metabolic derangement might well imply a coordinator role of their increased extracellular expressions at the onset of the host response in severe sepsis. Future studies are required to further advance our understanding of mechanisms that regulate the immune responses during severe sepsis and SIRS-related MOSF that may lead to the identification of new diagnostic, prognostic, or treatment targets.

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