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ORIGINAL ARTICLE

# Heat shock protein 27 in the pathogenesis of COVID-19 and non-COVID acute respiratory distress syndrome

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## Abstract

Acute respiratory distress syndrome (ARDS) is a common cause of hypoxemic respiratory failure in intensive care units that has increased dramatically as a result of the COVID-19 pandemic. In both COVID-19 and non-COVID ARDS, the pathogenesis of lung injury involves local (pulmonary) and systemic inflammation, leading to impaired gas exchange, requirement for mechanical ventilation, and a high risk of mortality. Heat shock protein 27 (HSP27) is a chaperone protein expressed in times of cell stress with roles in modulation of systemic inflammation via the NF-κB pathway. Given its important role as a modulator of inflammation, we sought to investigate the role of HSP27 and its associated auto-antibodies in ARDS caused by both SARS-CoV-2 and non-COVID etiologies. A total of 68 patients admitted to the intensive care unit with ARDS requiring mechanical ventilation were enrolled in a prospective, observational study that included 22 non-COVID-19 and 46 COVID-19 patients. Blood plasma levels of HSP27, anti-HSP27 auto-antibody (AAB), and cytokine profiles were measured on days 1 and 3 of ICU admission along with clinical outcome measures. Patients with COVID-19 ARDS displayed significantly higher levels of HSP27 in plasma, and a higher ratio of HSP27:AAB on both day 1 and day 3 of ICU admission. In patients with COVID-19, higher levels of circulating HSP27 and HSP27:AAB ratio were associated with a more severe systemic inflammatory response and adverse clinical outcomes including more severe hypoxemic respiratory failure. These findings implicate HSP27 as a marker of advanced pathogenesis of disease contributing to the dysregulated systemic inflammation and worse clinical outcomes in COVID-19 ARDS, and therefore may represent a potential therapeutic target.

**Keywords** Heat shock protein 27 · Heat shock protein 27 auto-antibody · Acute respiratory distress syndrome · COVID-19

## Introduction

Acute respiratory distress syndrome (ARDS) is a syndrome of diffuse lung injury involving local (pulmonary) and systemic inflammation. ARDS results in alveolar epithelial and endothelial injury, infiltration of alveolar airspaces with inflammatory exudate, culminating in impaired gas exchange (Swenson and Swenson 2021). ARDS can be precipitated by a variety of pulmonary and extra-pulmonary insults, including pneumonia, aspiration, sepsis, trauma, and many other causes (Swenson and Swenson 2021; Zambon and Vincent 2008). In recent years, SARS-CoV-2 (COVID-19) pneumonia has emerged as a common etiology of ARDS in ICUs worldwide, with a mortality rate of severe COVID-19 ARDS reported as high as 40% (Swenson and Swenson 2021; Zambon and Vincent 2008; Lim et al. 2021). Aside from a small number of immunomodulatory therapies approved for severe COVID-19, the management of ARDS remains primarily supportive with mechanical ventilation and intensive care. Further defining the pathogenic mechanisms of ARDS, including mechanisms that differentiate COVID-19 and non-COVID ARDS, may lead to novel targeted therapies for this deadly disease.

Heat shock protein 27 (HSP27) belongs to the small heat shock protein family. These proteins were first characterized by their robust response to cellular stresses and facilitation

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of refolding of damaged proteins (Vidyasagar et al. 2012). HSP27 has multiple roles in disease processes including cardiovascular disease, neuro-degenerative disease, cancer, and renal injury.

Upregulation of HSP27, HSP60, and HSP70 has been demonstrated in animal models of acute lung injury (Wheeler and Wong 2007). HSP27 is proposed to be involved in sepsis-associated acute lung injury via modulation of endothelial actin cytoskeleton, contributing to vascular permeability, immune cell infiltration, and alveolar edema (Hirano et al. 2004). In a rat model of LPS-induced lung inflammation, anti-TNF therapy reduced HSP27 phosphorylation and endothelial permeability suggesting cross talk between HSP27 and lung inflammation (Hirano et al. 2004). Furthermore, a role for HSP27 has been identified in inflammatory and fibrotic pulmonary processes including idiopathic pulmonary fibrosis (IPF) and extra-pulmonary diseases like renal tubulointerstitial fibrosis (Vidyasagar et al. 2012). These preclinical data implicate HSP27 as a modulator of pulmonary inflammation and acute lung injury, but its role in ARDS pathogenesis in humans is unknown.

Natural IgG auto-antibodies (AAB) to HSP27 are also found in humans, and the formation of HSP27-AAB immune complexes (ICs) in the circulation has been shown to modulate HSP27-induced inflammation via toll-like receptor 4 (TLR4) and NF-kB signaling pathways (Chiu et al. 2019; Shi et al. 2020). In cardiovascular disease, higher anti-HSP27 AAB is found to be protective against vascular disease (Chen et al. 2021). Vaccination of Apo E−/− mice with recombinant murine ortholog of HSP27 increased AAB levels, conferring a decrease in plaque inflammation and cholesterol levels (Chen et al. 2021). Sequestration of HSP27 by AAB was hypothesized to mediate these protective effects by blocking HSP27-induced inflammatory responses (Shi et al. 2020). The balance between HSP27 and anti-HSP27 AAB is proposed to be a determinant of HSP27-induced inflammation in disease. In ARDS, disrupting this balance (either by increased HSP27 or decreased AAB) may contribute to the dysregulated pulmonary and systemic inflammation.

The contribution of HSP27, and anti-HSP27 AAB, in ARDS remains unknown, and whether their contribution differs depending on the etiology (COVID-19 or non-COVID ARDS). Here, we compare patients admitted to ICU requiring mechanical ventilation for ARDS secondary to COVID-19 and non-COVID causes and find that patients with COVID-19 ARDS have HSP27 levels and a higher ratio of HSP27:AAB, which is associated with higher severity of illness, including more severe hypoxemic respiratory failure. This HSP27 response coincides with an exacerbated systemic cytokine storm, characterized by elevated levels of multiple inflammatory mediators including TNFα, GM-CSF, IL-10, and MCP-1.

## Methods

### Patient population

From September 2020 to January 2022, we prospectively enrolled patients with ARDS requiring mechanical ventilation from four intensive care units in Calgary, AB, Canada. Diagnosis of ARDS was based on the Berlin Definition (Ranieri et al. 2012) and independently confirmed by two intensive care specialists. New diagnosis of COVID-19 was determined via positive SARS-CoV-2 RT-PCR on endotracheal tube aspirate or nasopharyngeal swab at the time of admission. Patients were excluded with known pre-existing immune deficiency, chronic respiratory failure, neuromuscular disease, tracheostomy, blood hemoglobin < 9 g/dl, or goals of care that limited life-support interventions. Written informed consent was obtained from all patients or their most appropriate surrogate decision makers for critically ill patients who were unable to consent. This study received institutional research ethics approval from the University of Calgary and Alberta Health Services (REB18-1294 and REB20-0720).

### Clinical data

We collected baseline patient demographics and comorbidities from the patient's medical records. Patients were followed prospectively on day 1 and day 3 for the ratio of arterial oxygen partial pressure ($PaO_2$) to fractional inspired oxygen ($FIO_2$) P/F ratio and sequential organ failure assessment (SOFA) score. Duration of mechanical ventilation, ICU length of stay, hospital length of stay, thromboembolic complications, and 90-day all-cause mortality. Median follow-up time was 15 months.

### Biomarker analysis

Serial blood samples were collected on post enrollment day 1 and 3 while the patients remained in the ICU. Samples were centrifuged to generate platelet-depleted plasma and stored at − 80 °C for further analysis. Plasma HSP27 levels were measured as previously described (Seibert et al. 2013; Rayner et al. 2008), using an enzyme-linked immunosorbent assay kit specific to human HSP27 according to the manufacturer's instructions (QIA119, Calbiochem, San Diego, California). Assay detection range was 31.3 to 2000 pg/mL. Absorbance was measured at 450 nm with a microplate reader (Synergy Mx, BioTek, Winooski, VT, USA).

IgG anti-HSP27 AAB levels were measured using an ELISA developed in the O'Brien vascular lab (Shi et al. 2020). NUNC maxisorp plates (ThermoFisher) were coated with rHSP27 at a concentration of 500 ng/well in carbonate-bicarbonate buffer at room temperature for 12 h. Wells were blocked with 1% bovine serum albumin (BSA)/phosphate-buffered saline tween (PVST) and incubated with plasma at a final dilution of 1:2000 in 1% BSA followed for 2 h followed by 3 more washes in PBST. A horse radish peroxidase (HRP) labeled anti-human IgG (H&L) antibody #109–035-003, Jackson Immunoresearch, West Grove, PA) was used as a detection antibody at a dilution of 1:5000 and incubated for 1 h at RT. Finally, substrate solution (3,3′,3.5′-Tetramethylbenzidine Liquid Substrate, TMB; Millipore Sigma) was added to each well and incubated for 10 min avoiding direct light. The reaction was stopped by 2N $H_2SO_4$ and the optical density quantified at 450 nm using Synergy Mx plate reader (BioTek). Inflammatory cytokine measurements in plasma samples were performed by Eve Technologies (Calgary, AB, Canada) using a Human Cytokine Proinflammatory Discovery Assay, and reported as pg/mL for samples with detectable values.

### Statistical analysis

Data in figures are presented as median ± interquartile range, with values for each participant shown as dots on graphs. Statistical analyses were performed using non-parametric tests with Mann–Whitney *U* test (when comparing 2 groups) or a Kruskal–Wallis test with post hoc Dunn's test for multiple comparisons (when comparing >2 groups). Categorical variables are presented as number and percentages (%) and analyzed using Fisher's exact test. Differences were considered significant at $P$ values < 0.05 (two-tailed).

## Results

### Characteristics of patients with COVID-19 ARDS and non-COVID ARDS

A total of 68 patients with ARDS requiring mechanical ventilation were enrolled, including 46 patients with COVID-19 ARDS and 22 patients with ARDS caused by non-COVID etiologies (bacterial or non-SARS-CoV-2 viral pneumonia, septic shock, trauma, aspiration, pancreatitis, fat embolism

**Table 1** Clinical characteristics of patients with acute respiratory distress syndrome due to COVID-19 pneumonia and non-COVID causes. COVID-19 patients were also stratified into high and low HSP27 groups

| | Non-COVID ($N=22$) | COVID-19 ($N=46$) | $p$-value | COVID high HSP27 ($N=23$) | COVID low HSP27 ($N=23$) | $p$-value |
|---|---|---|---|---|---|---|
| Age in years (IQR) | 54 (18–86) | 59.5 (31–84) | 0.274 | 57 (31–84) | 62 (34–77) | 0.24 |
| Female (%) | 6 (27.3%) | 14 (30.4%) | >0.99 | 7 (30.4%) | 7 (30.4%) | >0.99 |
| History of cardiovascular disease | 6 (27.3%) | 8 (17.4%) | 0.356 | 4 (17.4%) | 4 (17.4%) | >0.99 |
| History of arterial clot | 1 (4.5%) | 4 (8.7%) | >0.99 | 2 (8.7%) | 2 (8.7%) | >0.99 |
| History of VTE | 0 | 1 (2.2%) | >0.99 | 0 | 1 (4.3%) | >0.99 |
| VTE risk factors | 2 (9.1%) | 3 (6.5%) | 0.656 | 1 (4.3%) | 2 (8.7%) | >0.99 |
| COVID-19 | 0 | 46 (100%) | n/a | 23 (100%) | 23 (100%) | n/a |
| Bacterial pneumonia | 9 (40.9%) | 0 | n/a | 0 | 0 | n/a |
| Viral pneumonia | 1 (4.5%) | 0 | n/a | 0 | 0 | n/a |
| Culture negative PNA | 9 (40.9%) | 0 | n/a | 0 | 0 | n/a |
| Septic shock | 4 (18.2%) | 0 | n/a | 0 | 0 | n/a |
| Aspiration | 4 (18.2%) | 0 | n/a | 0 | 0 | n/a |
| Esophageal rupture | 1 (4.5%) | 0 | n/a | 0 | 0 | n/a |
| Pancreatitis | 1 (4.5%) | 0 | n/a | 0 | 0 | n/a |
| Fat emboli | 1 (4.5%) | 0 | n/a | 0 | 0 | n/a |
| Trauma | 5 (22.7%) | 0 | n/a | 0 | 0 | n/a |
| Antibiotics | 22 (100%) | 46 (100%) | n/a | 23 (100%) | 23 (100%) | n/a |
| Dexamethasone | 0 | 45 (97.8%) | n/a | 22 (95.7%) | 23 (100%) | >0.99 |
| Remdesivir | 0 | 2 (4.3%) | n/a | 1 (4.3%) | 1 (4.3%) | >0.99 |
| Tociluzimab | 0 | 15 (32.6%) | n/a | 6 (26.1%) | 9 (39.1%) | >0.99 |

**Table 2** Clinical outcomes and day 1 and day 3 characteristics and heat shock protein 27 and autoantibody levels of acute respiratory distress syndrome. Groups are separated into non-COVID and COVID groups. Data are presented as median with range

| | Non-COVID ($N=22$) | COVID-19 ($N=46$) | $p$-value | COVID high HSP27 ($N=23$) | COVID low HSP27 ($N=23$) | $p$-value |
|---|---|---|---|---|---|---|
| SOFA day 1 | 8 (2–16) | 4 (2–15) | 0.0006 | 5 (2–15) | 4 (2–10) | 0.248 |
| SOFA day 3 | 8.5 (2–22) | 4 (1–11) | 0.01 | 4 (2–11) | 3 (1–10) | 0.265 |
| P/F ratio day 1 | 196.5 (138–360) | 170 (72–290) | 0.009 | 150 (72–265) | 182 (84–290) | 0.02 |
| P/F ratio day 3 | 217.5 (130–390) | 186 (62–348) | 0.014 | 171 (66–263) | 192 (62–348) | 0.13 |
| Arterial thrombus | 1(4.5%) | 1 (2.2%) | 0.55 | 1 (4.3%) | 0 | > 0.99 |
| VTE | 2 (9%) | 9 (19.7%) | 0.48 | 6 (26.1%) | 3 (13.0%) | 0.45 |
| Duration of mechanical ventilation | 7 (1–20) | 11.5 (2–75) | 0.028 | 11 (2–60) | 12 (02– 75) | 0.71 |
| ICU length of stay (to 90 days) | 9.5 (4–30) | 14 (3–89) | 0.062 | 14 (4–60) | 16 (3–89) | 0.85 |
| Hospital length of stay (to 90 days) | 18.5 (4–90) | 25 (7–90) | 0.175 | 25 (8–95) | 25 (7–90) | 0.83 |
| Mortality — number (%) | 7 (31.8) | 9 (19.6%) | 0.36 | 5 (21.7%) | 4 (19.4%) | > 0.99 |
| HSP27 levels pg/mL day 1 | 393.3 (34.9–840) | 502.8 (5.3–1893) | 0.01 | 632 (505.5–1893) | 433.3 (5.3–500) | < 0.0001 |
| HSP27 levels pg/mL day 3 | 350 (206.7–806.7) | 564 (101.3–1890) | < 0.0001 | 678.0 (341.3–1893) | 501.9 (101.3–957.3) | 0.016 |
| HSP27 AAB OD 450 nm day 1 | 0.18 (0.13–0.35) | 0.19 (0.08–2.61) | 0.96 | 0.19 (0.08–2.61) | 0.18 (0.08–0.82) | 0.80 |
| HSP27 AAB OD 450 nm day 3 | 0.19 (0.12–0.46) | 0.2 (0.06–2.24) | 0.85 | 0.19 (0.06–2.24) | 0.19 (0.06–0.45) | 0.78 |
| HSP27:AAB ratio day 1 | 1899 (266–4560) | 2542 (37–12836) | 0.06 | 3550 (246–12836) | 2136 (37–4364) | 0.002 |
| HSP27:AAB ratio day 3 | 1715 (617–4119) | 3085 (152–10645) | 0.003 | 3738 (152–10645) | 2157 (295–5838) | 0.044 |

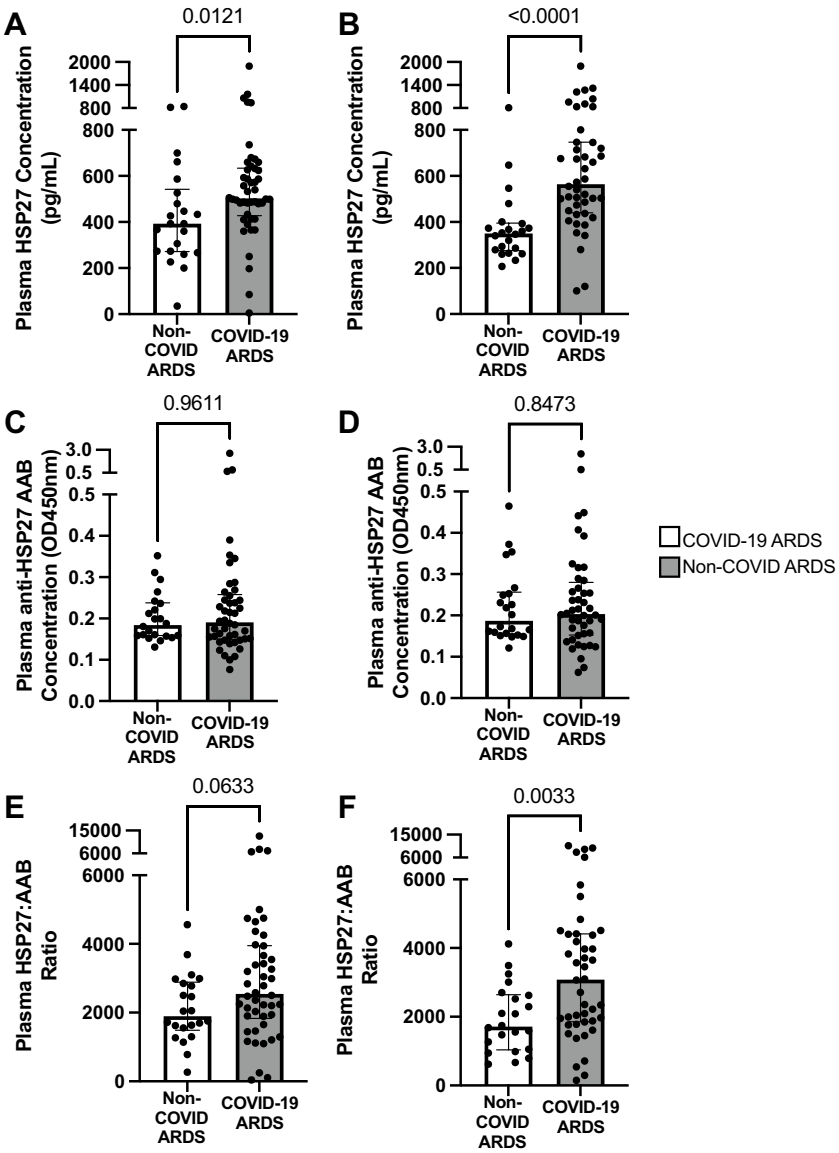


**Fig. 1** Comparison of plasma heat shock protein 27 (HSP27) in patients with acute respiratory distress syndrome (ARDS) caused by COVID-19 and non-COVID etiologies. Plasma was collected from patients with COVID-19 and non-COVID ARDS on days 1 and 3 of ICU admission to measure the levels of (**A**, **B**) HSP27 (**A** day 1, **B** day 3), (**C**, **D**) anti-HSP27 autoantibody (AAB) (**C** day 1, **D** day 3), and (**E**, **F**) the ratio of plasma HSP27:AAB (**E** day 1, **F** day 3). Statistical analysis using Mann–Whitney *U* test, *p* values as shown

syndrome, etc.; with some patients experiencing multiple potential etiologic diagnoses) (Table 1). Baseline demographics between cohorts were similar including age; comorbidities such as cardiovascular disease, thromboembolic disease; and were predominately male (Table 1). Patients with COVID-19 ARDS received targeted treatment interventions (dexamethasone in 97.8%, tocilizumab in 32.6%, and remdesvir in 4.3%). Otherwise, all patients in both groups were treated in the intensive care unit with mechanical ventilation, systemic antibiotics, VTE prophylaxis, and enteral nutrition (Table 1).

Consistent with the underlying diagnoses, patients with non-COVID ARDS had a higher degree of multi-organ dysfunction on both days 1 and 3 of ICU admission (sequential organ failure assessment [SOFA] score 8 vs 4 and 8.5 vs 4; $p=0.0006$ and $p=0.01$) (Table 2). However, patients with COVID-19 ARDS had higher severity of hypoxemic respiratory failure demonstrated by a reduced $PaO_2/FiO_2$ ratio on day 1 (170 vs 196.5; $p=0.009$) which persisted to day 3 (186 vs 217.5; $p=0.014$) (Table 2). This translated to a trend towards longer duration of mechanical ventilation (11.5 vs 7 days; $p=0.028$) and a longer duration of ICU length of stay (14 vs 9.5 days; $p=0.062$) for patients with COVID-19 ARDS (Table 2). No significant differences were noted in 90-day mortality or risk of arterial or venous thromboembolism (Table 2).

## HSP27 and AAB levels in patients with COVID-19 ARDS and non-COVID ARDS

Levels of circulating HSP27 were higher in patients with COVID-19 compared to non-COVID ARDS on days 1 and 3 (median 502.8 vs 393.3 pg/mL, $p=0.01$, and 564.0 vs 350.0 µg/mL, $p<0.0001$) (Fig. 1A, B; Table 2). Next, we measured the levels of anti-HSP27 AAB and observed no significant difference between COVID-19 and non-COVID

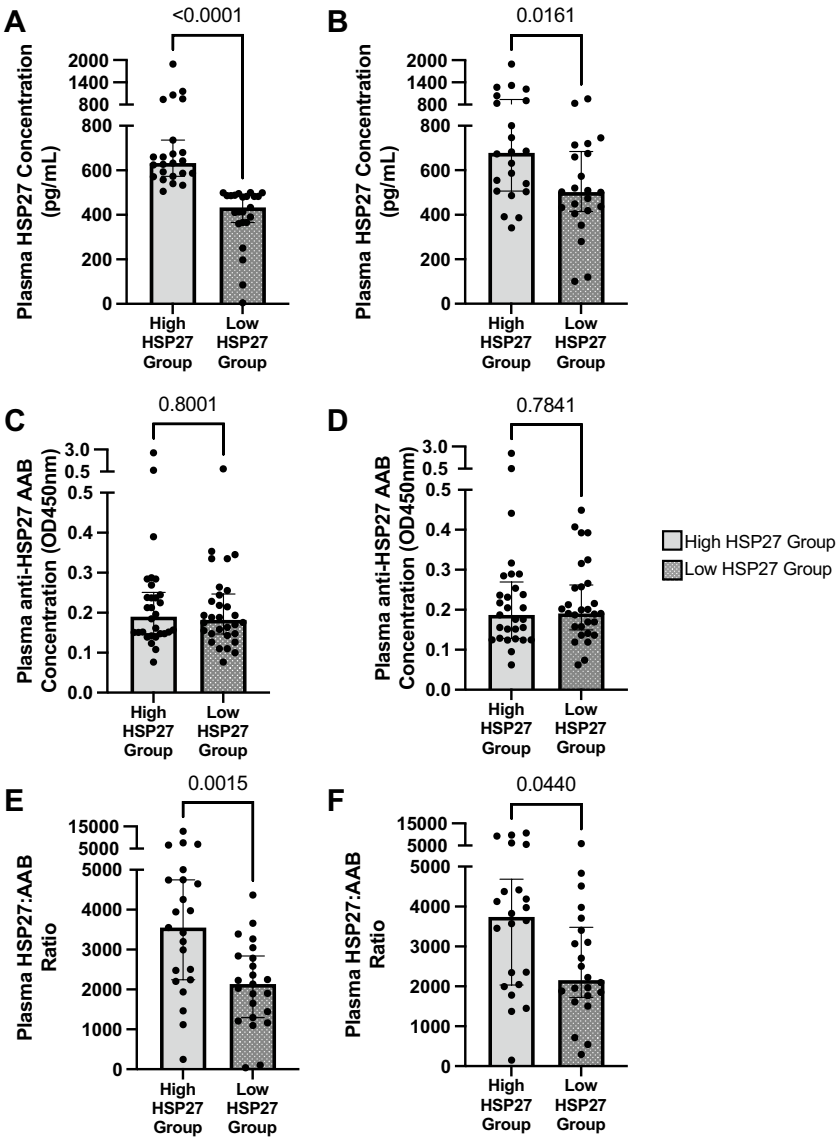


**Fig. 2** Plasma heat shock protein 27 (HSP27) and AAB levels in patients with COVID-19 ARDS stratified by high versus low circulating HSP27. COVID-19 patients were stratified into two groups based on plasma HSP27 levels at admission (high = above cohort median, low = below cohort median). Levels of **A**, **B** HSP27 (**A** day 1, **B** day 3), **C**, **D** anti-HSP27 autoantibody (AAB) (**C** day 1, **D** day 3), and **E**, **F** the ratio of plasma HSP27:AAB (**E** day 1, **F** day 3). Statistical analysis using Mann–Whitney *U* test, *p* values as shown

patients at either timepoint (median OD450 nm 0.19 vs 0.18, $p = 0.961$; and 0.20 vs. 0.19, $p = 0.847$) (Fig. 1C, D; Table 2). As a result, the ratio of HSP27:AAB levels trended higher in patients with COVID-19 ARDS, with a significant increased found on day 3 of ICU admission (day 1 median 2542 vs 1899, $p = 0.063$; and day 3 median 3085 vs 1715, $p = 0.003$) (Fig. 1E, F; Table 2). Collectively, these results demonstrate higher HSP27 levels without modulation of AAB levels, yielding higher effective circulating HSP27 concentrations in COVID-19 ARDS compared to non-COVID ARDS.

## Clinical outcomes in patients with COVID-19 ARDS stratified by HSP27 levels

Given that HSP27 appears to be differentially regulated in COVID-19 ARDS, we hypothesized that circulating HSP27 concentrations (or ratio of HSP27:AAB) may be prognostically informative to identify patients at increased risk of adverse outcomes. Using their day 1 HSP27 concentration, COVID-19 ARDS patients were stratified into high ($n = 23$) and low ($n = 23$) HSP27 based on whether they were higher or lower than to the population median (503 µg/mL). Of note, patient demographics and treatment characteristics were similar between HSP27-high and HSP27-low groups (Table 1). The median plasma concentrations of HSP27 for patients in the high and low group were 632.0 vs 433.3 pg/mL on day 1 ($p < 0.0001$) and 678.0 vs 501.9 µg/mL on day 3 ($p = 0.016$) respectively (Fig. 2A, B). There were no significant differences between the anti-HSP27 AAB levels (Fig. 2C, D). HSP27/AAB ratio was increased in the high HSP27 group compared to the low HSP27 group on both day 1 and day 3 (3550 vs 2136; $p = 0.0015$ and 3738 vs 2157; $p = 0.044$) (Fig. 2E, F).

**Table 3** Cytokine levels in patients with acute respiratory distress syndrome due to COVID-19 pneumonia and non-COVID causes. COVID-19 group was then stratified into high and low HSP27 groups. Data show median concentration in pg/mL and range, analyzed by Mann-Whitney U test (N=18-46 per group)

| | Non-COVID | COVID -19 | *p*-value | High HSP27 | Low HSP27 | *p*-value |
|---|---|---|---|---|---|---|
| GM-CSF (day 1) | 0.16 (0–24.6) | 5.080 (0–403.1) | 0.009 | 22.1 (0–403.1) | 0 (0– 9.6) | 0.0002 |
| GM-CSF (day 3) | 0.17 (0–19.2) | 0.00 (0–318.3) | > 0.99 | 0 (0–318.3) | 0 (0–129.9) | > 0.99 |
| IFNγ (day 1) | 3.91 (0.2–570.7) | 9.56 (0.4–595.1) | > 0.99 | 8.7 (0.8–595.1) | 11.3 (0.4–166.1) | > 0.99 |
| IFNγ (day 3) | 4.24 (0.1–110.6) | 13.11 (0.4–432.4)) | > 0.99 | 14.9 (0.5–432.4) | 12.2 (0.4–210) | > 0.99 |
| IL-1β (day 1) | 0.28 (0–1628) | 16.99 (0.64–282.7) | < 0.0001 | 15.3 (0.6–282.7) | 19.2 (3.9–175.6) | > 0.99 |
| IL-1β (day 3) | 0.16 (0–1448) | 17.63 (0.7–244.2) | < 0.0001 | 14.0 (0.6–244.2) | 20.6 (3–164.4) | > 0.99 |
| IL-2 (day 1) | 0.4 (0.03–219.9) | 0.7 (0–21.5) | > 0.99 | 0.9 (0–21.5) | 0.7 (0–16.6) | > 0.99 |
| IL-2 (day 3) | 0.5 (0.15–219.4) | 0.9 (0–17.7) | > 0.99 | 0.7 (0–17.7) | 0.9 (0–14.8) | > 0.99 |
| IL-4 (day 1) | 0.1 (0–51.8) | 0 (0–20.5) | 0.465 | 0 (0–3.8) | 0 (0–20.5) | > 0.99 |
| IL-4 (day 3) | 0.04 (0–52.6) | 0 (0–12.4) | > 0.99 | 0 (0–3.3) | 0 (0–12.4) | > 0.99 |
| IL-5 (day 1) | 0.5 (0–644.2) | 2.2 (0.3–45) | 0.572 | 2.8 (0.7–23.3) | 1.5 (0.3–45) | 0.748 |
| IL-5 (day 3) | 2.7 (0–1422) | 4.8 (0.3–84.1) | 0.689 | 7.2 (0.4–84.1) | 4.1 (0.3–83.8) | > 0.99 |
| IL-6 (day 1) | 106.7 (4.85–2096) | 82.6 (2.7–1932) | > 0.99 | 43.4 (2.7–1935) | 100.4 (5.9–1516) | > 0.99 |
| IL-6 (day 3) | 49 (0.8–1978) | 219.5 (0.6–7290) | 0.019 | 139.5 (1.7–7291) | 93.9 (0–1957) | > 0.99 |
| IL-8 (day 1) | 25.8 (3.9–1785) | 29.8 (5.2–1459) | > 0.99 | 33.0 (9.6–1459) | 27.3 (5.2–114.5) | 0.519 |
| IL-8 (day 3) | 16.1 (3.4–656.4) | 23.5 (1.3–417.6) | > 0.99 | 32.4 (11.7–176.1) | 16.8 (1.3–417.6) | 0.137 |
| IL-10 (day 1) | 3.8 (0–277.2) | 32.7 (3–1225) | 0.004 | 115.3 (12.4–1225) | 24.9 (4.4–394.4) | 0.011 |
| IL-10 (day 3) | 2.4 (0.4–96.4) | 25.9 (1.4–390.3) | 0.002 | 25.9 (2.2–312.1) | 23.1 (1.4–390.3) | > 0.99 |
| IL-12p70 (day 1) | 0.3 (0.02–807.7) | 1.2 (0–63) | > 0.99 | 1.4 (0–63) | 1.2 (0–56.9) | > 0.99 |
| IL-12p70 (day 3) | 0.3 (0–792.5) | 0.9 (0–44.3) | 0.918 | 0.7 (0–44.3) | 1.2 (0–17.9) | > 0.99 |
| IL-13 (day 1) | 1.3 (0–82.3) | 32.9 (0–754.1) | < 0.0001 | 27.2 (0–754.1) | 37.3 (0–397.7) | > 0.99 |
| IL-13 (day 3) | 0.8 (0–97.6) | 31.4 (0–917.2) | 0.0001 | 25.3 (0–719.2) | 47.3 (0–388.2) | > 0.99 |
| MCP-1 (day 1) | 357 (55.6–2966) | 434.6 (46.9–4360) | > 0.99 | 430.5 (51.4–1678) | 434.6 (46.9–4360) | > 0.99 |
| MCP-1 (day 3) | 183.2 (34–2728) | 615 (124.7–15,046) | 0.0007 | 1175 (520.2–15,046) | 408.2 (124.7–4019) | 0.0002 |
| TNFα (day 1) | 8.1 (1.5–482.2) | 104.7 (13.1–600.4) | 0.0001 | 123.7 (52.3–600.4) | 82.6 (11.9–276.9) | 0.009 |
| TNFα (day 3) | 7.2 (1.5–302.1) | 114.9 (13.8–653.6) | < 0.0001 | 132.4 (13.8–653.4) | 108.6 (23–434.6) | > 0.99 |

Next, we investigated the impact of HSP27 stratification on clinical outcomes in patients with COVID-19 ARDS. Patients in the high HSP27 strata (HSP27-high group) had more severe hypoxemia demonstrated by reduced PaO2/FiO2 ratios (150.0 vs 182.0; $p = 0.02$). Otherwise, patients in the high and low HSP27 strata had similar rates of thrombotic complications, duration of mechanical ventilation, ICU length of stay, and mortality. Collectively, these findings reveal that HSP27 concentrations and HSP27:AAB ratios were higher in COVID-19-related ARDS compared to non-COVID ARDS, and that in patients with COVID-19, the presence of higher HSP27 levels was associated with more severe hypoxemic respiratory failure.

## Impact of circulating HSP27 and anti-HSP27 AAB on systemic inflammatory responses in COVID-19 and non-COVID-19 ARDS

To investigate the relationships between circulating HSP27 and AAB on the systemic inflammatory response during ARDS, we performed multiplexed quantification of 13 key cytokines/chemokines in the plasma of patients on days 1 and 3 of ICU admission. Compared to non-COVID ARDS, patients with COVID-19 were found to have significantly higher levels of TNFα, IL-1β, IL-10, GM-CSF, and IL-13 on day 1 (Table 3; Fig. 3). By day 3 of ICU admission, patients with COVID-19 ARDS had higher levels of IL-6, MCP-1, as well as persistently higher levels of TNFα, IL-1β, IL-10, and IL-13 (Table 3). No significant differences were observed in the levels of IFNγ, IL-2, IL-4, IL-5, IL-8, and IL-12p70 between patients with COVID-19 and non-COVID ARDS.

Next, we investigated the correlations between plasma HSP27 concentrations and cytokine levels across all patients. Heatmap visualization of the Spearman correlation coefficients demonstrated striking positive correlations between HSP27 concentrations and plasma levels of key inflammatory cytokines including IL-1β, TNFα, IL-10, MCP-1, GM-CSF, and IL-2, with the magnitude of correlations being stronger on day 3 than day 1 (Fig. 3A). Having observed this relationship between HSP27 and the systemic cytokine storm, we next investigated whether patients stratified into high HSP27

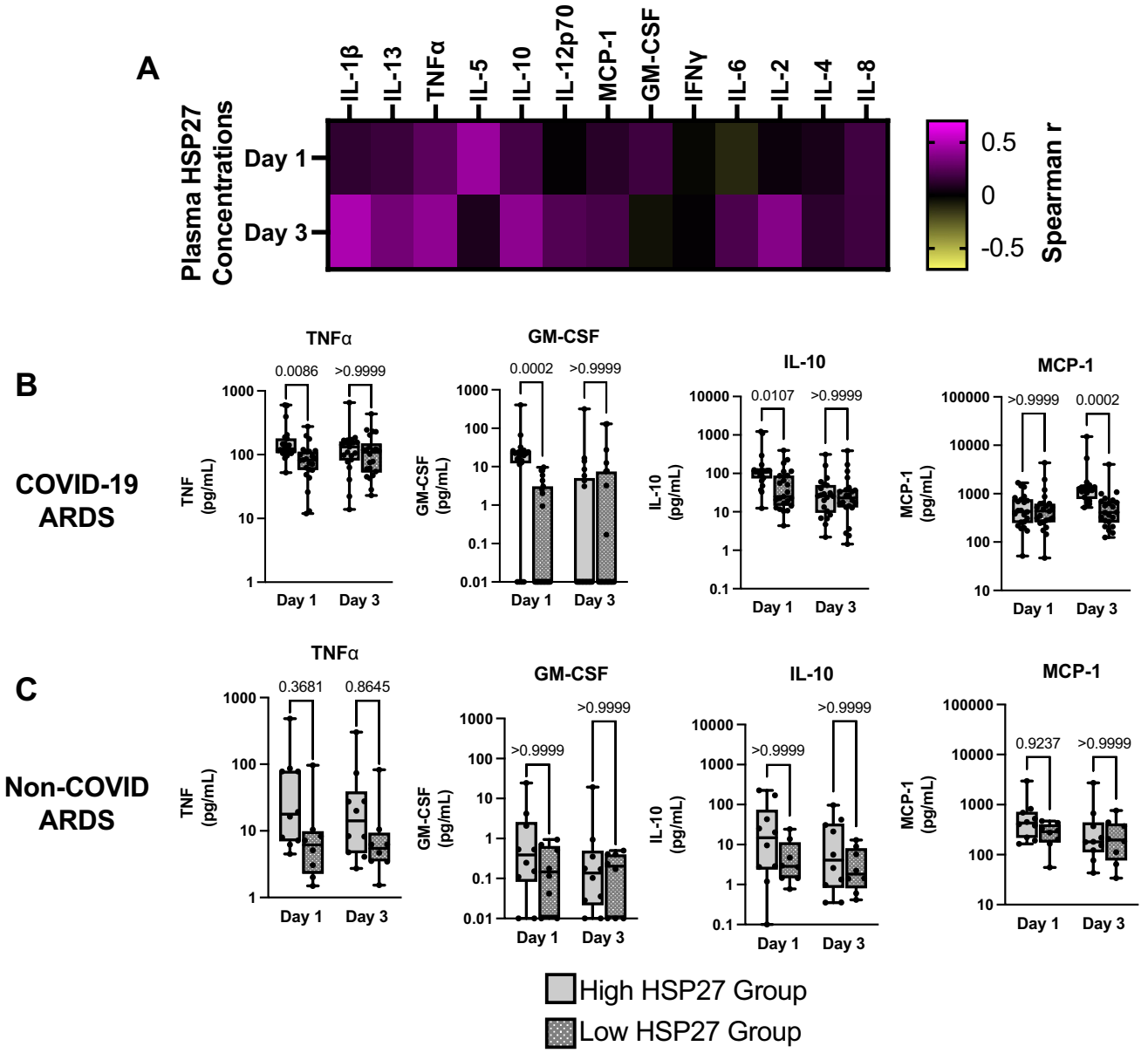


**Fig. 3** Circulating HSP27 levels and systemic cytokine responses in ARDS. **A** Heatmap showing the Spearman correlation coefficient between plasma HSP27 concentrations and the concentrations of circulating inflammatory mediators in patients with COVID-19 on days 1 and 3 of ICU admission. **B** Comparison of key circulating cytokine levels on days 1 and 3 of ICU admission in COVID-19 ARDS patients with high versus low HSP27 levels on admission (high = above cohort median, low = below cohort median). **C** Comparison of key circulating cytokine levels on days1 and 3 of ICU admission in non-COVID ARDS patients with high versus low HSP27 levels on admission (high = above cohort median, low = below cohort median). Statistical analysis using Kruskal–Wallis test with post hoc Dunn's test for multiple comparisons, *p* values as shown

versus low HSP27 groups mounted different plasma cytokine responses for both COVID-19 and non-COVID ARDS. In patients with COVID-19 ARDS, HSP27 high group displayed significantly higher levels of TNFα, GM-CSF, and IL-10 on day 1, with higher levels of MCP-1 on day 3 (Fig. 3B). In contrast, we did not identify any significant differences in plasma cytokine levels in non-COVID ARDS patients with high versus low HSP27 stratification (Fig. 3C). Collectively, these data demonstrate a unique systemic cytokine response in patients with COVID-19 ARDS, wherein higher levels of HSP27 are associated with a more robust inflammatory response.

## Discussion

In this study, we found that circulating HSP27 and HSP27:AAB ratio are elevated in the acute phase of COVID-19 ARDS compared to non-COVID ARDS patients. Higher

levels of circulating HSP27 on admission were found to be persistent over the initial 3 days of critical illness and were associated with adverse clinical outcomes including more severe hypoxemia. These adverse clinical outcomes in COVID-19 patients with higher levels of circulating HSP27 were coupled with a more robust systemic inflammatory response, with elevated levels of multiple inflammatory cytokines. Collectively, these data implicate HSP27 as a potential pathological mediator linked to worse inflammation and adverse outcomes in patients with COVID-19 ARDS.

Our findings also align with a prior study of HSP27 levels in 245 patients with COVID-19-related respiratory failure, which reported elevated HSP27 levels were associated with an increased need for supplemental oxygen and mechanical ventilation (Wendt et al. 2021). However, this study did not investigate whether the role of HSP27 was specific to COVID-19 or generalizable to other forms of severe pneumonia or ARDS, nor did it investigate putative linkages with inflammatory pathogenesis. It is now well established that clinical outcomes differ between patients with ARDS caused by COVID-19 versus non-COVID etiologies (as we also observed in our cohorts), including more severe hypoxemia and a longer median duration of mechanical ventilation (Bain et al. 2021). This is coupled to emerging evidence demonstrating clear differences in the immunopathogenesis of ARDS in patients with COVID-19 versus non-COVID causes (Panda et al. 2022; Sinha et al. 2022). Consistent with this, we observed marked differences in HSP27 levels between patients with COVID-19 ARDS compared to non-COVID ARDS, and this translated into distinct systemic inflammatory responses and its association with circulating HSP27 concentrations.

Aside from intrinsic chaperone activity, HSP27 has been found to have pleiotropic effects such as an anti-apoptotic factor and an important regulator of inflammatory cytokine production via modulation of NF-κB signaling (Liu et al. 2015; Arrigo 2007). Circulating HSP27 has been shown to directly induce production of multiple proinflammatory mediators in mouse models. For example, administration of recombinant HSP27 (rHSP27) to atherosclerotic prone Apo $E^{-/-}$ mice induced upregulation of 24 genes with both inflammatory and anti-inflammatory cytokines and chemokines, including IL-1β, IL-8, TNF-α, Il-6, L-10, interferon, and GM-CSF (Rayner et al. 2008; Salari et al. 2013). Furthermore, the inflammatory potential of circulating HSP27 is modulated by anti-HSP27 AAB which can bind to form immune complexes that neutralize the function of HSP27 and induce alternate immunomodulatory signaling (Chiu et al. 2019; Shi et al. 2020). We found that elevation of circulating HSP27 in patients with ARDS was independent of changes in AAB levels (which were unchanged), thus yielding an elevated ratio of HSP27:AAB and proinflammatory cytokine response. As HSP27 is expressed in times of cell stress, such as lung injury, there is clearly a disproportionate release of HSP27 without a compensatory AAB response, resulting in an imbalance in HSP27 to AAB that may contribute to the dysregulated inflammatory response in COVID-19 ARDS.

Intracellular HSP27 has a role in antioxidant response, anti-apoptosis, and cytoskeletal architecture (Batulan et al. 2016). Extracellular small heat shock protein release has been detected in human serum in a variety of disease processes and non-disease processes such as excessive exercise (Lancaster and Febbraio 2005). Increased blood HSP27 levels are found in patients with chronic pancreatitis (Liao et al. 2009), malignancy (Huang et al. 2010), insulin resistance (Pengiran Burut et al. 2010), and flares of multiple sclerosis (Ce et al. 2011) but are reduced with cardiovascular disease — particularly coronary artery disease (Batulan et al. 2016). While there is no leader sequence and therefore conventional export from cells, it is now recognized that exosomes play an important role in transporting HSP27 out of cells (Batulan et al. 2016). In vitro models with kidney cells, HSP27 laden exosomes have been demonstrated to increase IL-10 release and stimulated NF-κB activation (Shi et al. 2019). Further research will be required to determine whether HSP27-laden extracellular vesicles play a role in ARDS.

Higher levels of HSP27 in patients with COVID-19 were associated with increases in both pro-inflammatory (TNFα, GM-CSP, MCP-1) and immunomodulatory (IL-10) cytokines. IL-10 is a pleiotropic immunomodulatory cytokine primarily known for its anti-inflammatory effects both in innate and adaptive immunity (Islam et al. 2021). The cytokine storm of ARDS and critical illness is characterized by simultaneous elevation of both pro-inflammatory and immune-modulatory/anti-inflammatory cytokines, reflecting a widespread state of immune dysregulation that is associated with worse outcomes (van der Poll et al. 2021; Fajgenbaum and June 2020). Interestingly, prior studies of patients with COVID-19 have also reported elevated IL-10 levels in those with severe disease, which was associated with adverse outcomes (Han et al. 2020; Zhao et al. 2020).

Current COVID therapies to modulate the cytokine storm include an IL-6 inhibitor, tocilizumab (TCZ), found to improve clinical outcomes including survival in critically ill patients (Zhang et al. 2020). In vivo models suggest that TCZ modulates the inflammatory profile of monocytes, with decreased expression of IL-8, IL-6, and MCP-1 and decreased neutrophil extracellular traps (NETs) (Ruiz-Limón et al. 2017). In our small study, we did not find differences between HSP27 and anti-HPS27 AAB levels in patients treated with TCZ or remdesivir. Furthermore, we did not observe a relationship between HSP27 levels and circulating IL-6, suggesting differential pathways of inflammatory pathogenesis. Given that HSP27 activity seems to be

uncoupled from IL-6, targeting HSP27 therapeutically may yield additive benefits to current therapies like TCZ. Our lab has previously demonstrated favorable activity of HSP27 neutralization using immunization to boost the production of anti-HSP27 antibodies on mouse models of atherosclerotic disease (Chen et al. 2021). Therefore, HSP27 may represent an avenue for novel therapeutic development for ARDS.

This exploratory study has several limitations. First, the overall sample size of this study was modest and had subtle differences in patient characteristics between groups, with non-COVID ARDS patients being slightly younger with a higher SOFA score. However, this is likely a true reflection of differential disease characteristics between COVID-19 and non-COVID ARDS, rather than a by-product of modest sample size, and is therefore unlikely to confound our findings (e.g., patients with non-COVID ARDS due to trauma are typically younger with higher burden of multisystem injury). Detailed information on pre-existing comorbidities and medications were unavailable, and therefore, we were unable to analyze their potential impact on HSP27 levels. Furthermore, while the patients in this study were balanced with respect to key treatment interventions for ARDS (antibiotics, sedation, mechanical ventilation, VTE prophylaxis, enteral nutrition), disease-specific treatments were administered to patients with COVID-19 (dexamethasone, tocilizumab, remdesvir). As these treatment covariables are exclusive to patients with COVID-19, we cannot uncouple their potential impact on HSP27 or AAB levels between those with COVID-19 and non-COVID ARDS. Lastly, due to limitations of sample volume, we were unable to directly measure HSP27-AAB immune complexes and therefore relied on the surrogate of HSP:AAB ratio as previously described (Chen et al. 2021).

Overall, our results support a pathological role for circulating HSP27 in the inflammatory pathogenesis and clinical outcomes of critically ill patients ARDS, with a disproportionate impact on COVID-19 compared to non-COVID ARDS. These findings would support further research to investigate the potential of HSP27 as a biomarker for disease severity as well as a putative treatment target for patients with ARDS.

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**Data Availability** All data required to interpret the results of this study are included in the manuscript. Datasets are available upon request.

## Declarations

**Competing interests** The authors declare no competing interests.

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