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ORIGINAL ARTICLE

Gut-barrier, Endotoxemia, Cytokine, and Heat Shock Protein in Relation to Severity of Heat-Related Illness in Emergency Department; A Comparative Study

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Abstract: **Introduction:** Heat-related illness severity is judged by bedside indicators that do not capture gut injury, endotoxin burden, or the stress response. This study aimed to evaluate the association between admission gut-barrier, endotoxemia, cytokine, and heat shock protein levels with severity of heat-related illness. **Methods:** This prospective comparative study was conducted in three emergency departments, in Baku, Azerbaijan during 2025 heatwave season. Adult patients who were referred following a heat-related illness were categorized into three groups (based on clinical type) of heat stroke, heat exhaustion, and controls. Then the admission levels of 7 biomarkers (intestinal fatty acid-binding protein (I-FABP), Zonulin, lipopolysaccharide (LPS), sCD14, IL-6, TNF-α, and HSP70) were compared between groups. In addition, the association between biomarker levels and severity of heat stroke (three or more failing organ systems at admission, derived from SOFA) was evaluated. Biomarkers were measured at the first emergency blood draw and, in heat stroke, at 6 and 24 hours. **Results:** The study comprised 300 participants including 150 cases with heat stroke (85 (56.7%) classic and 65 (43.3%) exertional), 75 cases with heat exhaustion, and 75 controls. All seven markers rose stepwise across groups (trend $p < 0.001$); adjusted I-FABP and LPS were 4.0-fold (95% CI: 3.2–5.0) and 4.9-fold (95% CI: 4.0–6.0) the control levels and correlated ($\rho = 0.75$). For grade 3 severity of heat stroke, I-FABP and LPS increased the area under the curve (AUC) from 0.69 to 0.82 over age, core temperature and cooling delay ($p = 0.001$), from 0.78 to 0.85 over lactate (DeLong $p = 0.009$; bootstrap $p = 0.154$), and not over lactate, procalcitonin and CRP (0.87 to 0.87). The gut–cytoprotection mismatch index (GCMI) was associated with 28-day mortality (19.3%; AUC: 0.76) but added nothing to SOFA (odds ratio: 0.89; $p = 0.795$). After adjustment for age, creatinine, and baseline concentration, slower 24-h clearance in non-survivors persisted for HSP70 only ($p = 0.023$). Each hour of cooling delay carried 11% higher I-FABP and 10% higher LPS. **Conclusion:** Gut injury, endotoxemia and a weaker HSP70 response tracked heat-related illness and heat stroke severity but added little beyond procalcitonin and SOFA. The study contributes same-patient marker profiles with 24-h kinetics; the panel is hypothesis-generating and needs testing against strong comparators in external cohorts.

Keywords: Heat Stroke; Fatty Acid-Binding Proteins; Endotoxemia; HSP70 Heat-Shock Proteins; Biomarkers

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1. Introduction

Heat-related illness is an expanding burden on health systems. The 2025 Lancet countdown estimated an average of 546,000 heat-related deaths per year between 2012 and 2021 (1), and heat-related mortality in people older than 65 years in 2023 was 167% above the 1990s level (2). Europe alone recorded an estimated 61,672 heat-related deaths in the summer of 2022 (3). Exposure is also rising in the South Caucasus: mean annual temperature in Azerbaijan has increased, and heatwaves are projected to lengthen and intensify, with the lowland plains most affected (4).

Emergency departments (EDs) in these regions will likely experience an increase in patients presenting with heat-related illnesses. These cases may range in type from mild conditions, such as heat rash, to complete medical emergencies, such as heat stroke.

Heat stroke is defined by a core temperature above 40 °C with central nervous system dysfunction (5, 6); classic heat stroke

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follows passive exposure and exertional heat stroke occurs during strenuous activity (5, 6). Intensive-care unit (ICU) mortality reaches 26.5% in exertional and 63.2% in classic heat stroke (6).

Severity assessment in these patients relies on bedside indicators and organ-failure scores that do not measure gut injury, endotoxin burden or the stress response, and it is unclear whether these signals relate to severity or add to routine markers (lactate, C-reactive protein [CRP], procalcitonin).

In critical illness, splanchnic hypoperfusion, epithelial death and junction failure let microbial products enter the circulation and provoke systemic inflammation (7); in heat stroke, direct thermal injury adds to this ischemic insult (8).

Lim proposed that endotoxin (lipopolysaccharide (LPS)) leaving the gut can reach a threshold that triggers systemic inflammation, termed heat sepsis (9). Because no single test captures barrier function (10), measurable readouts are needed.

Intestinal fatty acid-binding protein (I-FABP) is released from damaged enterocytes, and higher admission I-FABP was associated with 28-day mortality in septic shock and/or acute respiratory distress syndrome (11).

Heat shock protein 70 (HSP70), a molecular chaperone, protects cells under stress (12). Evidence in heat stroke is incomplete and mechanistic work comes mainly from animal and cell models (8), including worse heat-induced death of hypothalamic cells when HSP72 is reduced (13).

Plasma I-FABP and diamine oxidase were linked to severity in one clinical study (14), but gut injury and endotoxemia do not always move together: endotoxin did not correlate with I-FABP in gram-positive septic shock (15), and baseline barrier markers did not differ between exertional heat illness cases and matched controls (16).

A review lists I-FABP and extracellular HSP72 as biomarkers of heat stroke-induced organ injury (17), while heat stroke prognostic models rely on routine clinical variables (18, 19). Markers are usually examined singly, at one time point, without HSP70 and rarely against established indicators.

Based on the points mentioned above, this study aimed to evaluate the association between admission gut-barrier, endotoxemia, cytokine, and heat shock protein levels with severity of heat-related illness.

2. Methods

2.1. *Study design and setting*

This prospective multicenter comparative study enrolled patients in the emergency departments of three tertiary-care hospitals (Liv Bona Dea Hospital, Məlhəm Beynəlxalq Hospital and Central Customs Hospital) in Baku, Azerbaijan, during the 2025 heatwave season (May–September). Patients were categorized into three groups (based on clinical type) of heat stroke, heat exhaustion, and controls. Then the admission level of 7 biomarkers of I-FABP, Zonulin, LPS, sCD14, IL-6, TNF-α, and HSP70 were compared between groups. In addition, the association between biomarker levels and severity of heat stroke was evaluated.

The research questions were: do gut-barrier, endotoxemia, cytokine and HSP70 markers differ across the three groups?; is I-FABP associated with LPS, and does LPS statistically explain part of the link to organ dysfunction?; do admission I-FABP and LPS classify concurrent grade 3 (most severe) heat stroke beyond age, core temperature and cooling delay, and beyond lactate, CRP and procalcitonin?; does HSP70, alone or within a mismatch index (GCMI), add value for severity, and is the index associated with 28-day mortality and early deterioration beyond the Sequential Organ Failure Assessment (SOFA) score?; do 0-6-24 hours kinetics differ by severity and 28-day mortality?; and is longer onset-to-cooling time associated with higher gut injury and endotoxemia?

The design followed the Gut-Endotoxin-Cytoprotection Mismatch (GECM) framework (Figure 1): gut injury (I-FABP, Zonulin), endotoxin translocation (LPS, soluble CD14 [sCD14]), cytokines (interleukin-6 [IL-6], tumor necrosis factor-α [TNF-α]) and the cytoprotective response (HSP70) were measured in the same patients across three groups, with 24-hour follow-up in heat stroke. Data were recorded on standardized case report forms under study-committee supervision. Admission data were complete; the only missing biomarker values were the 24-hours samples of five patients who died earlier.

The study was approved by the institutional review board of Azerbaijan Medical University (No. 2025-0712) and conducted according to the Declaration of Helsinki. Patients with impaired consciousness were enrolled with consent from an authorized representative, and re-consent was obtained after recovery where required; other participants gave written informed consent. Reporting followed the STROBE statement.

2.2. *Participants*

Adults (≥18 years) were enrolled consecutively during the season: 360 were screened, 35 were ineligible and 25 declined to consent, leaving 300 participants. The 300 participants (150 heat stroke: 85 classic, 65 exertional; 75 heat exhaustion; 75 controls) came from three sites (Site 1: Liv Bona Dea Hospital with 130 cases; Site 2: Məlhəm Beynəlxalq Hospital with 85 cases; Site 3: Central Customs Hospital with 85 cases).

Patients with inflammatory bowel disease, recent gastrointestinal surgery, advanced chronic liver disease, immunosuppression, hematological malignancy, antibiotics in the previous 7 days, pregnancy, central nervous system infection, thyroid storm, neuroleptic malignant syndrome, drug intoxication, and refusal of consent were excluded.

2.3. *Data gathering*

Trained staff recorded demographics, comorbidity, occupation, acclimatization, exposure, vital signs, Glasgow Coma Scale (GCS), and laboratory results. Core temperature was measured rectally on arrival. Heat stroke patients received evaporative cooling, ice-water immersion, or ice packs with cold intravenous fluids; heat exhaustion patients received oral rehydration or evaporative cooling. Cooling method was recorded but not used as a covariate.

Organ dysfunction was scored with the SOFA from admission data (six systems; renal sub-score from creatinine only; cardiovascular sub-score 1 for mean arterial pressure <70 mmHg, 3 for norepinephrine ≤0.1 μg/kg/min and 4 for >0.1 μg/kg/min). A sub-score ≥2 defined dysfunction. Recorded totals differed by one point from totals recomputed from the components in 18 of 150 patients with heat stroke; no grade changed (Table S1).

Grades 1, 2 and 3 corresponded to 0–1, 2 and ≥3 dysfunctional systems at admission; grade 3 was the primary outcome. The grade is derived from admission SOFA and overlaps with the definition of heat stroke through its central nervous system

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component, so it is a concurrent severity measure and not a validated scale or independent outcome.

2.4. *Definitions*

Heat stroke was defined as rectal core temperature ≥40.0 °C with central nervous system dysfunction after environmental or exertional heat exposure, other causes excluded, classified as classic or exertional.

Heat exhaustion was heat-related symptoms with a core temperature above 37.5 °C and up to 39.9 °C and a GCS score of 15.

Controls were asymptomatic outdoor workers (core temperature ≤37.5 °C), recruited on the same days and frequency-matched to the heat exhaustion group on sex and age (±10 years).

Onset-to-cooling time (cooling delay) was the interval from symptom onset to the start of active cooling.

Door-to-cooling time was the interval from arrival to the start of active cooling.

Prognostic endpoints were 28-day mortality, early deterioration (SOFA rise ≥2 points from admission to the 72-h maximum) and intensive care unit (ICU) admission; the last two were defined post hoc. Maximum 72-h SOFA was the outcome of the mediation models.

2.5. *Blood sampling, assays, and derived variables*

Venous blood was drawn into pyrogen-free tubes at admission (T0) in all participants (once in controls) and, in heat stroke, at 6 hours (T6) and 24 hours (T24). In heat stroke, T0 was drawn a median of 35 min (interquartile range 21–48; maximum 60) after arrival and door-to-cooling time was 17 min (10–23), so 133 of 150 T0 samples followed the start of cooling; sampling delay was therefore a covariate in the group comparison and a sensitivity covariate in the other models.

Samples were separated within 1 h and stored at −80 °C until batch analysis. I-FABP, zonulin, HSP70, sCD14, IL-6 and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA) and LPS by a chromogenic Limulus amebocyte lysate (LAL) assay (plasma for LPS, serum for all other assays) in six batches. LAL spike recovery was within the 85–115% acceptance range, and IL-6 intra- and inter-assay coefficients of variation were 5.2% and 8.1%. Manufacturers, catalog numbers, quantification limits and coefficients of variation (CVs) of all kits are in Table S2. Eleven I-FABP and two LPS values (T6 or T24) were below the lower limit and were analyzed as measured; replacing them by the limit did not change the clearance comparison (Table S1). All samples of one patient were run in one batch and every batch contained all three groups; batch was unrelated to site (χ^2 p = 0.32) and group (p = 0.55) and weakly related to admission month (p = 0.057).

Concentrations were right-skewed and were ln-transformed. For each marker, the batch effect was estimated from a regression of ln concentration on group, age, sex, body mass index (BMI) and batch, subtracted from every value (centered on the mean batch effect) and applied unchanged to T6 and T24. Batch-adjusted values were used in all analyses except the group ANCOVA, which included batch as a covariate.

Two derived variables used fixed formulas: the gut–cytoprotection mismatch index (GCMI) = z(ln I-FABP) + z(ln LPS) − z(ln HSP70), and clearance = ln(T24/T0), with ln(T6/T0) for the early window. The z-scores used heat stroke cohort means and standard deviations (SDs) of batch-adjusted ln values (I-FABP 7.55 and 0.86; LPS −1.17 and 0.64; HSP70 1.64 and 0.61), so the index can be recomputed in other samples.

2.6. *Statistical analysis*

Precision was examined by the Hanley–McNeil method: 20 severe and 32 non-severe patients give 80% power (two-sided α = 0.05) to detect an area under the curve (AUC) of 0.80 against 0.50. The 58 grade 3 and 92 grade 1–2 patients gave over 95% power for an AUC of 0.75 and about 10 events per predictor in six-predictor models. Only 29 deaths and 30 early deteriorations occurred, so these models were limited to one to four predictors (at least 7 events per predictor).

Analyses were scripted in Python 3.13.18. A fixed seed (20260101) made repeated runs identical. Tests were two-sided with α = 0.05. Continuous data are median (interquartile range) and categorical data n (%). Assumptions, batch and site effects, and collinearity were audited (Table S3).

Group comparison. Ln admission values were analyzed by ANCOVA adjusted for age, sex, BMI, ELISA batch and sampling delay (HC3 standard errors) and expressed as adjusted geometric mean ratios, with Benjamini–Hochberg adjustment across the seven biomarkers. The expected order (control < heat exhaustion < heat stroke) was tested by Jonckheere–Terpstra, and pairwise contrasts by Dunn tests (Holm). Exertional and classic heat stroke were compared by adjusted regression.

Gut injury, endotoxin, and organ dysfunction. In heat stroke, the I-FABP–LPS association was estimated by Spearman correlation and by partial correlation adjusted for age, core temperature and cooling delay. The indirect effect of ln I-FABP on maximum SOFA through ln LPS came from linear path models with the same covariates (5,000 bootstrap resamples; BCa interval), repeated with admission SOFA and with subtype, comorbidity and site added. Because I-FABP, LPS and SOFA were measured at one visit, this was an exploratory decomposition and not causal mediation; robustness was examined by E-value and sensitivity models.

Discrimination and incremental value. Grade 3 was modeled by logistic regression: M0 (age, core temperature, cooling delay), M1 (M0 + I-FABP + LPS), M2 (M1 + HSP70) and M3 (M0 + GCMI), with markers as standardized ln values. Two stronger comparators were added after the primary analysis: M0L (M0 + lactate) and M0P (M0L + procalcitonin + CRP), each extended with I-FABP and LPS (M1L, M1P). GCS, blood pressure, creatinine and admission SOFA were not entered because they define the grade. Covariates were fixed by clinical reasoning without p-value-based selection. AUCs followed DeLong; because the DeLong test is conservative for nested models, differences were also tested by an out-of-bag paired bootstrap (2,000 resamples) and likelihood-ratio tests, and both p-values are reported. Optimism, calibration, cross-validation, leave-one-site-out transportability, and collinearity were also assessed.

Kinetics. Ln marker values at T0, T6 and T24 were analyzed by linear mixed models with a patient-level random intercept, adjusted for age; grade × time and mortality × time interactions were tested by likelihood-ratio tests. Clearance was compared across grades (Jonckheere–Terpstra) and between survivors and non-survivors (Mann–Whitney), and additionally by linear regression adjusted for age, ln creatinine and ln baseline concentration (HC3 standard errors), because fold change is coupled to baseline and I-FABP may depend on renal function. Missing 24-h samples were examined by complete-case, last-observation-carried-forward and best-case analyses.

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Cooling delay. Ln I-FABP and ln LPS were regressed on onset-to-cooling time (per hour), adjusted for core temperature and age, with HC3 standard errors. Non-linearity was assessed with a restricted cubic spline, and sensitivity models were also fitted.

Prognostic endpoints. For 28-day death, early deterioration, and ICU admission, AUCs of admission SOFA, lactate, procalcitonin, CRP, creatinine and the biomarkers were compared (DeLong). The incremental value of GCMI was tested over SOFA plus age, and over SOFA, age, and CRP, by likelihood-ratio, DeLong and out-of-bag bootstrap tests. Associations used Firth penalized logistic regression, time to death an age-adjusted Cox model, and survival across GCMI tertiles Kaplan–Meier curves with the log-rank test.

Multiplicity and reproducibility. Multiplicity was handled within families (seven biomarkers, Benjamini–Hochberg; pairwise contrasts, Holm); other analyses addressed one stated question each and were not adjusted further, so secondary, post hoc and sensitivity results are supportive only.

3. Results

3.1. *Baseline characteristics of studied patients*

The study comprised 300 participants including 150 cases with heat stroke (85 (56.7%) classic and 65 (43.3%) exertional), 75 cases with heat exhaustion, and 75 controls. Table 1 compares the baseline characteristics of patients between three groups. Sex (p = 0.864), BMI

(p = 0.388) and site (p = 0.863) did not differ significantly across groups. Heat stroke patients were older (median 49 (interquartile range (IQR): 29–63)) years,) than the controls (34 (IQR: 28–40)) and heat exhaustion patients (33 (IQR: 26–39)), more often hypertensive (24.0% vs. 5.3% and 8.0%, respectively) or diabetic (16.0% vs. 5.3% and 0%, respectively), and had less home cooling and shorter acclimatization (6 vs. 21 and 9 days, respectively). Age showed the largest imbalance (SMD = 0.94), so group comparisons were adjusted for age, sex, BMI, ELISA batch and sampling delay.

Median core temperature was 41.2, 38.7 and 37.0 °C in heat stroke, heat exhaustion and controls. In heat stroke, median GCS was 10 (IQR: 8–12), median admission SOFA score 6 (IQR: 5–9), and onset-to-cooling time was 202 (IQR: 71–340) minutes (against 43 (27–55) minutes in heat exhaustion). 53 (35.3%) heat stroke cases had severity grade 1, 39 (26.0%) grade 2, and 58 (38.7%) grade 3. 80 (53.3%) heat stroke cases required intensive care unit admission and 29 (19.3%) died within 28 days.

3.2. *Admission biomarkers across groups*

All seven biomarkers rose stepwise from controls to heat exhaustion and heat stroke (Table 2; trend and FDR-adjusted p < 0.001; ε^2 0.38–0.62), with all pairwise Holm p < 0.001. After adjustment, I-FABP in heat stroke was 4.0 (95% CI: 3.2 to 5.0) times, LPS 4.9 (95% CI: 4.0 to 6.0) times, and HSP70 2.9 (95% CI: 2.4 to 3.6) times of control levels. Ratios for the other markers ranged from 1.7 (sCD14) to 15 (IL-6).

Controls and heat stroke occupied largely separate regions, with heat exhaustion between (Figure 2A).

Within heat stroke, all markers except HSP70 rose with grade, whereas HSP70 fell from grade 1 to 3 (mean z-score 1.1, 0.6, 0.1; Figure 2B). Exertional and classic heat stroke did not differ in any biomarker (adjusted ratios 0.97–1.76; Holm p ≥ 0.357), although the I-FABP interval was wide (1.00 to 3.12).

Figure 2C–E shows half-violin, box (median, interquartile range), and individual values on a log scale for admission level of I-FABP, LPS, and HSP70 in three groups.

3.3. *Intestinal injury, endotoxemia and organ dysfunction*

In the 150 patients with heat stroke, I-FABP and LPS were strongly correlated (Spearman ρ = 0.75, 95% CI: 0.65 to 0.82, on batch-adjusted values and 0.71 on raw concentrations; partial ρ = 0.68, 0.56 to 0.77; Figure 3A). Both correlated with maximum SOFA score (ρ = 0.66 and 0.67), whereas HSP70 correlated inversely with it (ρ = −0.65; Figure 3B).

In the exploratory path model (Table 3), the indirect effect of ln I-FABP on maximum SOFA through LPS was 1.12 points (BCa 95% CI: 0.49 to 1.77), 38.9% of the total effect of 2.87 (2.32 to 3.42); the direct effect was 1.76 (0.95 to 2.54; Figure 3C). Results were similar for admission SOFA (indirect effect 0.93, 0.34 to 1.55) and with subtype, comorbidity and site added (1.10, 0.50 to 1.79; 40.2% mediated). Because all three variables were measured at one visit, the order I-FABP → LPS → organ dysfunction cannot be established.

3.4. *Classification of concurrent grade 3 heat stroke against clinical comparators*

For grade 3 versus grades 1–2, core temperature discriminated poorly (AUC: 0.57, 95% CI: 0.47 to 0.66), lactate and CRP moderately (0.75, 0.66 to 0.82) and procalcitonin better (0.83, 0.75 to 0.89), whereas I-FABP and LPS each reached 0.78 (0.70 to 0.85), low HSP70 0.76 (0.68 to 0.83) and GCMI 0.81 (0.74 to 0.87; Table 4; Figure 4A). Admission SOFA is not compared because it defines grade 3.

Model M0 (age, core temperature, cooling delay) had an AUC of 0.69 (0.59 to 0.77). Adding I-FABP and LPS (M1) raised it to 0.82 (0.74 to 0.88; ΔAUC: 0.13, 0.05 to 0.21; DeLong p = 0.001; bootstrap interval 0.01 to 0.23, p = 0.032; optimism-corrected 0.67 and 0.80). With lactate in the base model (M0L, 0.78), I-FABP and LPS raised the AUC to 0.85 (ΔAUC: 0.07, 0.02 to 0.12; DeLong p = 0.009; bootstrap interval −0.03 to 0.14, p = 0.154). With lactate, procalcitonin and CRP (M0P, 0.87, 0.80 to 0.92) there was no gain (M1P 0.87; ΔAUC: 0.003, −0.011 to 0.017, p = 0.637; likelihood-ratio p = 0.403; Figure 4B).

In the model that also contained HSP70 (M2; Table 5), higher core temperature (adjusted OR 4.0 per 1 °C, 1.3 to 12.5) and lower HSP70 (0.31 per SD of ln value, 0.15 to 0.64) were associated with grade 3. I-FABP (1.75, 0.83 to 3.69) and LPS (1.48, 0.66 to 3.32) were not significant individually (variance inflation factors 3.1 and 3.7). Cooling delay was not associated with grade 3 (0.91 per hour, 0.76 to 1.09).

3.5. *Incremental value of HSP70 and the mismatch index*

Adding HSP70 to M1 (M2) gave an AUC of 0.85 (0.78 to 0.90; ΔAUC: 0.03, −0.01 to 0.06; DeLong p = 0.149; bootstrap p = 0.301), a small, non-significant gain, although the likelihood-ratio test favored the larger model (p < 0.001). Replacing the three marker terms by GCMI (M3) gave 0.84 (0.77 to 0.89; difference from M1 0.02; DeLong p = 0.094; bootstrap p = 0.093). GCMI was associated with grade 3 at an adjusted OR of 6.7 per SD (3.3 to 13.6), increased across grades (trend p < 0.001; Figure 4C), and grade 3 patients clustered where injury was high and HSP70 low (Figure 4D).

Hosmer–Lemeshow p was 0.070 for M2 and 0.784 for M3. The cross-validated calibration slope of M2 was 0.82 (0.53 to 1.11)

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and the Brier score 0.171, against 0.220 for M0 (Figure 5A). The bootstrap intervals were wider than the DeLong intervals (M1 vs M0 0.13, 0.01 to 0.23; M2 vs M1 0.03, −0.04 to 0.08; Table S1). Net benefit of M1 and M2 exceeded that of M0 across threshold probabilities of about 0.2 to 0.55 (Figure 5B), and the out-of-bag AUC gain from I-FABP and LPS exceeded that from HSP70 (Figure 5C). Leave-one-site-out AUCs for M2 were 0.80, 0.80 and 0.82 (Table S1).

3.6. *Biomarker kinetics over 24 hours*

By 24 hours, median I-FABP had fallen to 0.14, 0.20 and 0.48 of its admission values in grades 1 to 3, LPS to 0.37, 0.65 and 0.80 (after a rise to 1.74 at 6 h in grade 3), and HSP70 had risen 2.32-, 2.04- and 1.26-fold (all trend $p < 0.001$; Table 6). The grade-by-time interaction was significant for all three markers ($p < 0.001$; Figure 6A–C).

Unadjusted, non-survivors cleared I-FABP and LPS more slowly than survivors (24-h fold change 0.61 vs 0.22 and 1.23 vs 0.48; both $p < 0.001$), and HSP70 rose less (1.08 vs 2.01; $p < 0.001$); the mortality-by-time interaction was significant for all three markers ($p < 0.001$; Figure 6D–F). At 6 h, differences were significant for I-FABP ($p = 0.024$) and LPS ($p = 0.019$) but not HSP70 ($p = 0.214$; Figure 7A). After adjustment for age, creatinine, and baseline concentration (n = 145), the difference persisted for HSP70 (ratio 0.69, 0.50 to 0.95; $p = 0.023$) but not for I-FABP (1.27, 0.81 to 2.00; $p = 0.296$) or LPS (1.36, 0.90 to 2.05; $p = 0.142$). By grade 3, I-FABP clearance remained slower (1.52, 1.01 to 2.29; $p = 0.045$), whereas LPS (1.09) and HSP70 (0.82) did not differ (Table S1). The five patients who died within 24 h had no 24-h sample; unadjusted differences persisted under complete-case, last-observation-carried-forward and best-case analyses (Table S1).

3.7. *Cooling delay*

Each additional hour between onset and cooling was associated with 11% higher I-FABP (geometric mean ratio 1.11, 1.06 to 1.16) and 10% higher LPS (1.10, 1.06 to 1.15), after adjustment for age and core temperature (Table S1). The relationships were close to linear (spline $p = 0.519$ and 0.322; Figure 7B, C) and similar after adding subtype or pre-onset exposure time (Table S1). Door-to-cooling time was not associated with either marker ($p = 0.157$ and 0.176).

3.8. *Prognostic endpoints: death and early deterioration*

Mortality was 2 of 53 (3.8%) in grade 1, 4 of 39 (10%) in grade 2 and 23 of 58 (40%) in grade 3. For 28-day death, admission SOFA (AUC: 0.85, 0.76 to 0.91) and procalcitonin (0.86, 0.77 to 0.92) discriminated at least as well as I-FABP (0.76), LPS (0.73), low HSP70 (0.71) and GCMI (0.76, 0.65 to 0.85); lactate (0.67), CRP (0.64) and core temperature (0.59) were lower. Firth odds ratios per SD were 2.90 (1.81 to 5.01) for I-FABP, 2.60 (1.61 to 4.51) for LPS, 0.48 (0.29 to 0.75) for HSP70 and 3.00 (1.82 to 5.36) for GCMI; the age-adjusted Cox hazard ratio for GCMI was 1.75 per SD (1.19 to 2.57; $p = 0.005$). Deaths were 2 of 50, 8 of 50 and 19 of 50 in the lowest, middle, and highest GCMI tertiles (log-rank $\chi^2 = 20.0$, $p < 0.001$; Figure 7D). GCMI added nothing to SOFA and age (AUC: 0.888 with and without GCMI; odds ratio: 0.89, 0.38 to 2.08; $p = 0.795$; unchanged with CRP added, Table S1), so its association with death is not independent of organ dysfunction already captured by SOFA.

Early deterioration (SOFA rise ≥2 points; 30 events) was identified poorly by admission SOFA (AUC: 0.68, 0.57 to 0.78) and better by GCMI (0.81, 0.71 to 0.88), CRP (0.83, 0.73 to 0.90), I-FABP (0.74) and LPS (0.74). Adding GCMI to SOFA and age raised the AUC from 0.68 to 0.82 (ΔAUC: 0.14, 0.04 to 0.24; DeLong $p = 0.007$; bootstrap $p = 0.070$; odds ratio: 5.4 per SD, 2.4 to 12.0); with CRP in the base model, from 0.83 to 0.85 ($p = 0.359$; odds ratio: 3.4, 1.5 to 8.1). For ICU admission (80 events), SOFA gave 0.89 and GCMI added nothing (odds ratio: 1.36; $p = 0.391$; Table S1).

3.9. *Sensitivity analyses*

Mediation estimates were unchanged by adding subtype or explicit batch terms, and a negative-binomial model was consistent (E-value 2.06; 1.57 for the confidence limit; Table S1). M2 reached an AUC of 0.85 with subtype and 0.86 with batch terms (biomarker likelihood-ratio $p < 0.001$). Sampling delay (median 17 min after cooling began, IQR: 6–30) was unrelated to grade 3 (odds ratio: 0.90 per 10 min, 0.68 to 1.20) and to I-FABP, LPS and HSP70 (ρ −0.03 to 0.04; all $p > 0.6$). With it added, M0 and M1 had AUCs of 0.68 and 0.82 (bootstrap $p = 0.024$), the indirect effect was 1.15 (0.47 to 1.81; 40.0% mediated), and each hour of cooling delay carried 11% higher I-FABP and 10% higher LPS (Table S1).

4. Discussion

In 300 participants from Baku, gut injury, endotoxemia and a weaker HSP70 response tracked heat-related illness severity, but their added value over strong clinical comparators was limited. Earlier studies showed endotoxemia with TNF and IL-1α release in acute heat stroke (20), raised cytokines (21) and an association of plasma I-FABP and diamine oxidase with severity (14); IL-6 had the largest adjusted ratio here (15). Those studies examined one or two markers at one time point. This cohort adds markers of injury, translocation and cytoprotection in the same patients, a graded design with heat exhaustion and controls, 0–6–24 h kinetics, and an estimate of how cooling delay relates to marker burden.

Four findings go beyond confirmation. Low HSP70 relative to gut injury (GCMI) was associated with grade 3 and death, although HSP70 alone added little discrimination. Severe cases and non-survivors cleared the markers more slowly, although only some contrasts held after adjustment. Each hour of cooling delay was associated with about 10% higher I-FABP and LPS. The discrimination gain from I-FABP and LPS fell from 0.13 over a weak clinical model to nil over lactate, procalcitonin and CRP, and GCMI added nothing to SOFA for death. These are associations from three centers and 29 deaths, with severity graded at the same visit as the first sample.

4.1. *Gut injury and endotoxemia: a mechanistic reading*

Heat stroke injures the gut by splanchnic ischemia and direct thermal damage to enterocytes (8, 9). Hyperthermia opens epithelial tight junctions in cell culture and severe heat stress produces endotoxemia in rats and primates (22), the basis of the gastrointestinal paradigm of heat stroke (23, 24). Our data are compatible with this: I-FABP and LPS were tightly coupled ($\rho = 0.75$; partial $\rho = 0.68$), both rose stepwise across groups, and LPS accounted statistically for about 39% of the I-FABP–SOFA association, although one time point cannot show which came first. Coupling is not automatic: running raised I-FABP about 2.5-fold while LPS fell in healthy athletes (25), endotoxin did not follow I-FABP in gram-positive septic shock (15), and baseline I-FABP did not separate exertional heat illness cases from controls (16). A threshold effect (9) is one reading, since

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our patients (median 41.2 °C) were far above the volunteers' peak of 39.4 °C (25), but these data do not test it.

4.2. *Parallel routes beyond endotoxin*

The mediated share was partial: the direct path from I-FABP to SOFA (1.76 points) exceeded the indirect path through LPS (1.12), and an E-value of 2.06 (1.57 for the confidence limit) means that a modest unmeasured confounder could explain it (26). Experimental work points to parallel routes, including HMGB1 signaling through Toll-like receptor 4 (27, 28) and endothelial injury with coagulation activation (29, 30), none of which we measured; evidence for endotoxin and cytokines as mediators is largely correlative (31), as ours is.

4.3. *HSP70: protective reserve or danger signal?*

HSP70 behaved less simply than expected: higher in heat stroke than controls (adjusted ratio 2.9), yet falling from grade 1 to 3 and correlated inversely with maximum SOFA (ρ = −0.65). Intracellular HSP70 is protective (8, 12), and reducing HSP72 worsens heat-induced death of rat hypothalamic cells (13), whereas extracellular HSP70 can stimulate monocyte cytokine production through CD14, TLR2 and TLR4 (32, 33). A serum ELISA cannot separate the two, so GCMI is best read as an empirical composite and not as proof that serum HSP70 is protective.

HSP70 rose less by 24 h in grade 3 and in non-survivors (Table 6), consistent with a blunted stress response; in dogs with naturally occurring heat stroke the 24-h serum HSP72 profile also appeared to relate to survival (34), although its shape differed from ours.

4.4. *Discrimination, prognosis, and kinetics*

Adding I-FABP and LPS to age, core temperature and cooling delay raised the AUC for grade 3 from 0.69 to 0.82 (optimism-corrected 0.67 and 0.80), but M0 is a weak comparator: core temperature alone gave 0.57, perhaps because one arrival reading sits at a variable distance from onset (median cooling delay 202 min). The gain fell to 0.07 over lactate, with a bootstrap interval including zero, and to 0.003 over lactate, procalcitonin and CRP. Grade 3 is also derived from SOFA, so these analyses describe concurrent severity and not prediction. This agrees with the earlier link of I-FABP with heat stroke severity (14), but incremental value over routine markers was not shown. HSP70 added little discrimination (0.03; p = 0.149), and GCMI matched the three separate terms (0.84 vs 0.85), so the case for the index is parsimony and not superiority; its constants and cut-offs are cohort-specific and need external testing.

For prognosis, SOFA and procalcitonin (AUC: 0.85 and 0.86 for death) outperformed the biomarkers (0.73–0.76), and GCMI added nothing to SOFA and age (odds ratio: 0.89; p = 0.795). Published emergency-department models report AUCs of 0.77–0.81 for heat stroke mortality, with SOFA at 0.70 in one cohort (18), and recalibrated SOFA scores are under evaluation (19); any new marker must be judged against such comparators. GCMI did add to SOFA and age for early deterioration (AUC: 0.68 to 0.82) but much less over CRP (0.83 to 0.85); that post hoc endpoint had 30 events. The mortality findings are therefore exploratory associations and not evidence of better risk stratification.

I-FABP clears quickly (half-life about 11 min in a trauma cohort (35)), so the higher 24-h fold change in non-survivors could reflect continuing enterocyte loss, renal function or baseline level; after adjustment for age, creatinine and baseline, the differences by death were not significant for I-FABP and LPS (p = 0.296 and 0.142), whereas HSP70 by death (p = 0.023) and I-FABP by grade 3 (p = 0.045) persisted. The slower-clearance hypothesis is therefore only partly supported. LPS rose again at 6 h in grade 3 (1.74 times admission) and remained 1.23 times admission at 24 h in non-survivors, consistent with further translocation or weaker neutralization; these data cannot separate the two.

4.5. *Cooling delay*

Experimental heat stroke is quantified by heat load, temperature above a threshold multiplied by time (27), and cooling delay is a crude clinical proxy for exposure duration. The near-linear relationship with both markers (spline p = 0.519 and 0.322) fits longer exposure prolonging splanchnic ischemia and thermal injury. One 39-patient series of classic heat stroke reported lower mortality when cooling began within 60 min (15% vs 33%), not significant and uncontrolled (36). Delay was not associated with grade 3 (odds ratio: 0.91 per hour, 0.76 to 1.09), and sicker patients may have been cooled sooner or later for unrecorded reasons, so the association may be partly confounded. Door-to-cooling time showed no association, possibly because of its narrow range (median 17 min) or a relevant delay before arrival.

4.6. *Implications*

Risk stratification. Adding I-FABP and LPS to bedside variables improved discrimination of grade 3 (Table 4), but not beyond procalcitonin and CRP, and the mismatch index did not add to SOFA for death. A panel would need to show value over such comparators, most plausibly for early deterioration, in a new cohort.

Monitoring. If slower clearance in severe cases is confirmed after adjustment for renal function and baseline level, persistent I-FABP at 24 h could flag patients needing closer monitoring, which makes serial sampling a design requirement.

Cooling. Rapid cooling remains the priority (5, 6). The association between delay and marker levels supports recording onset-to-cooling time as a quality indicator, but does not show that earlier cooling lowers gut injury.

Subtypes and setting. Classic and exertional heat stroke did not differ significantly in any biomarker, although subgroups were small (85 and 65) and the I-FABP interval wide. Heat exposure is rising in the South Caucasus (4), so local data will be needed to calibrate any panel.

4.7. *Strengths and limitations*

Strengths are the prospective design with consecutive enrollment, markers of injury, translocation and cytoprotection in the same patients, two comparison groups, serial sampling, covariates fixed in advance, comparison with lactate, CRP, procalcitonin and SOFA, internal validation, and reproducible code.

This was an observational study, so associations and the exploratory mediation estimate are not causal, and severity and biomarkers came from the same visit. Most T0 samples (133 of 150) followed the start of cooling (median 35 vs 17 min), so admission levels may differ from pre-cooling values; adjustment for sampling delay did not change the estimates (Table S1), although only 17 samples preceded cooling. Controls were heat-exposed workers and not emergency patients, so the

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group gradient is the least informative finding (spectrum bias), and heat stroke patients were older (median 49 vs 33–34 years), leaving residual confounding by age possible. The grade is study-specific and derived from admission SOFA; early deterioration, ICU admission and the lactate, procalcitonin and CRP comparators were added post hoc, and creatinine and consciousness level, which define the grade, were not used as comparators. Onset time rests on patient or bystander report, and earlier cooling may reflect milder illness. With 29 deaths and 30 early deteriorations, models were small and intervals wide. GCMI is standardized within this cohort, so its constants and cut-offs are sample-derived and validation was internal. LAL matrix controls were limited to spike recovery and CVs of three assays were not available, so absolute EU/mL values are best read as relative levels. No sample-size calculation preceded enrollment, and screening numbers were available only in total. Barrier function has no single test (10); we had no permeability probe, tissue data or HMGB1, coagulation or endothelial markers, so mechanistic statements are interpretations. All centers were in one country, and selection bias is possible: 25 eligible patients declined, representatives consented for unconscious patients, and only tertiary-care cases were enrolled.

5. Conclusions

Gut injury, endotoxemia and a weaker HSP70 response tracked the severity of heat-related illness at admission in this three-center emergency study. I-FABP and LPS were closely coupled ($\rho = 0.75$), cleared more slowly in severe cases and rose with cooling delay. However, the apparent discrimination gain from I-FABP and LPS shrank from 0.13 over a weak clinical model to 0.07 over lactate and to none over lactate, procalcitonin and CRP, and the mismatch index added nothing to SOFA for 28-day death. The severity grade was derived from SOFA and the first sample came from the same visit, so these findings describe concurrent severity and not prediction; mediation was only exploratory, and clearance differences were only partly robust to adjustment. The panel is therefore hypothesis-generating only. It merits testing in external cohorts against strong clinical comparators, with pre-cooling sampling, documented assay quality control and a rapid point-of-care assay, before any clinical use.

6. Declarations

6.1. *Acknowledgments*

We thank the emergency department staff, laboratory teams and study participants at the three participating hospitals in Baku for their support.

6.2. *Conflict of interests*

The authors declare no conflict of interest.

6.3. *Funding and supports*

This research received no specific funding.

6.4. *Authors' contributions*

All authors fulfilled the four ICMJE authorship criteria, including substantial contributions to the conception or design of the work or the acquisition, analysis, or interpretation of data; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work.

6.5. *Ethical statement*

The study was approved by the institutional review board of Azerbaijan Medical University (No. 2025-0712) and was conducted in accordance with the Declaration of Helsinki. Reporting followed the STROBE statement.

6.6. *Informed consent*

Informed consent was obtained from all participants before any data were recorded. Participants with decision-making capacity gave written informed consent. For patients with impaired consciousness, consent was obtained from an authorized representative before data recording, and re-consent was obtained after recovery where required.

6.7. *Availability of supporting data*

The data supporting the findings of this study are available from the corresponding author on reasonable request.

6.8. *Using artificial intelligence chatbots statement*

During the preparation of this work, the authors used an AI tool only for proofreading and grammar correction. The authors reviewed and edited all content and take full responsibility for the published article.

6.9. *Other declarations*

Author contributions (CRediT) All authors contributed to the study conception and design, data collection, analysis and interpretation, and manuscript drafting. All authors read and approved the final manuscript.

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Table 1: Comparing the baseline characteristics and outcomes between the study groups

Characteristic	Control (n = 75)	Heat exhaustion (n = 75)	Heat stroke (n = 150)	P	Max SMD
Demographics					
Age, years	34 (28–40)	33 (26–39)	49 (29–63)	<0.001	0.94
Male sex	55 (73.3)	52 (69.3)	107 (71.3)	0.864	0.09
Body mass index, kg/m^2	26.1 (22.9–28.4)	26.1 (22.2–28.1)	26.1 (23.4–29.4)	0.388	0.22
Comorbidity					
Hypertension	4 (5.3)	6 (8.0)	36 (24.0)	<0.001	0.55
Diabetes mellitus	4 (5.3)	0 (0.0)	24 (16.0)	<0.001	0.62
Chronic kidney disease	0 (0.0)	3 (4.0)	8 (5.3)	0.147	0.34
Cardiovascular disease	0 (0.0)	2 (2.7)	11 (7.3)	0.024	0.40
Diuretic or anticholinergic use	0 (0.0)	2 (2.7)	16 (10.7)	0.002	0.49
No home cooling access	28 (37.3)	44 (58.7)	98 (65.3)	<0.001	0.58
Prior heat illness	4 (5.3)	12 (16.0)	14 (9.3)	0.087	0.35
Acclimatization, days	21 (17–25)	9 (6–13)	6 (2–11)	<0.001	2.13
Environment					
Wet-bulb globe temperature, °C	33.4 (30.8–34.9)	33.9 (31.3–35.5)	34.5 (32.3–35.9)	0.032	0.38
Exposure time, hour					
Median (IQR)	5.6 (4.3–6.8)	4.4 (3.1–5.5)	16.7 (5.6–34.1)	<0.001	1.30
Presentation vital signs					
Core temperature, °C	37.0 (36.8–37.1)	38.7 (38.5–39.0)	41.2 (40.8–41.5)	<0.001	10.86
Glasgow Coma Scale score	15 (15–15)	15 (15–15)	10 (8–12)	<0.001	3.08
Mean arterial pressure, mmHg	90 (84–96)	92 (87–96)	82 (75–90)	<0.001	0.85
Lactate, mmol/L					
Median (IQR)	—	1.6 (1.4–1.8)	2.4 (1.7–3.2)	<0.001	1.07
Cooling time					
Onset-to-cooling, min	—	43 (27–55)	202 (71–340)	<0.001	1.35
Door-to-cooling, min	—	12 (10–22)	17 (10–23)	0.036	0.31
Organ dysfunction and outcome					
SOFA score at admission	—	0 (0–1)	6 (5–9)	<0.001	2.58
Maximum SOFA score, 0–72 h	—	0 (0–1)	7 (5–10)	<0.001	2.62
ICU admission	—	0 (0.0)	80 (53.3)	<0.001	1.51
28-day mortality	—	0 (0.0)	29 (19.3)	<0.001	0.69
Enrollment site					
Liv Bona Dea Hospital	31 (41.3)	33 (44.0)	66 (44.0)		
Məlhəm Beynəlxalq Hospital	21 (28.0)	24 (32.0)	40 (26.7)	0.863	—
Central Customs Hospital	23 (30.7)	18 (24.0)	44 (29.3)		
Type of heat stroke					
Classic	—	—	85 (56.7)	—	—
Exertional	—	—	65 (43.3)		
Severity grade					
1	—	—	53 (35.3)	—	—
2	—	—	39 (26.0)	—	—
3	—	—	58 (38.7)	—	—

Continuous variables: median (interquartile range); Kruskal–Wallis test (three groups) or Mann–Whitney U test (two groups with data). Categorical variables: n (%); chi-square or Fisher/permutation exact test. Max SMD = largest pairwise standardized mean difference; values >0.10 indicate imbalance, which is why age, sex, body mass index, ELISA batch and sampling delay enter all group comparisons as covariates. — = not collected or not applicable. SOFA: Sequential Organ Failure Assessment.

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Table 2: Admission gut-barrier, endotoxemia, cytokine and heat shock protein levels by study group

Biomarker	Control (n = 75)	Exhaustion (n = 75)	Stroke (n = 150)	Exhaustion vs. control	Stroke vs. control	Stroke vs. exhaustion	ε^2
I-FABP (pg/mL)	407 (279–598)	841 (515–1,113)	1,869 (1,118–3,329)	1.89 (1.51–2.36)	4.00 (3.18–5.03)	2.12 (1.72–2.61)	0.48
Zonulin (ng/mL)	33.3 (25.2–44.5)	46.0 (33.4–57.1)	69.5 (52.2–91.6)	1.44 (1.28–1.62)	2.04 (1.82–2.29)	1.42 (1.28–1.57)	0.39
LPS (EU/mL)	0.06 (0.04–0.08)	0.13 (0.08–0.18)	0.32 (0.20–0.46)	2.12 (1.71–2.63)	4.87 (3.96–5.99)	2.30 (1.91–2.75)	0.57
sCD14 (µg/mL)	1.85 (1.47–2.20)	2.41 (1.92–2.73)	3.31 (2.58–3.99)	1.27 (1.15–1.41)	1.67 (1.52–1.84)	1.32 (1.20–1.44)	0.42
IL-6 (pg/mL)	2.70 (1.35–4.45)	7.90 (5.35–14.8)	46.0 (24.2–86.0)	3.61 (2.66–4.90)	15.07 (10.97–20.70)	4.18 (3.14–5.55)	0.62
TNF-α (pg/mL)	3.60 (2.55–4.85)	6.40 (4.60–9.45)	14.4 (8.62–22.1)	1.84 (1.52–2.21)	3.56 (2.91–4.35)	1.94 (1.61–2.34)	0.47
HSP70 (ng/mL)	1.99 (1.27–2.60)	2.74 (2.17–4.64)	5.04 (3.31–7.72)	1.60 (1.33–1.92)	2.94 (2.43–3.56)	1.84 (1.55–2.18)	0.38

Median (interquartile range). Adjusted geometric mean ratios (95% CI) from ANCOVA on ln-transformed values with age, sex, body mass index, ELISA batch and sampling delay as covariates (HC3 robust SE). ε^2: Kruskal–Wallis effect size. For every marker, the Jonckheere–Terpstra trend test (control < heat exhaustion < heat stroke) and the omnibus ANCOVA group effect (Benjamini–Hochberg FDR across seven markers) gave $p < 0.001$. I-FABP: intestinal fatty acid-binding protein; LPS: lipopolysaccharide (endotoxin); sCD14: soluble CD14; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; HSP70: heat shock protein 70.

Table 3: Indirect pathway from intestinal injury to organ dysfunction through endotoxemia

Outcome	a: I-FABP → LPS	b: LPS → SOFA	c′: direct effect	c: total effect	Indirect effect (a × b)	p (indirect)	Mediated, %
Maximum SOFA, 0–72 h (primary)	0.55 (0.46–0.63)	2.02 (0.92–3.17)	1.76 (0.95–2.54)	2.87 (2.32–3.42)	1.12 (0.49–1.77)	<0.001	38.9
SOFA at admission (sensitivity)	0.55 (0.46–0.63)	1.68 (0.61–2.75)	1.62 (0.86–2.39)	2.55 (2.08–3.03)	0.93 (0.34–1.55)	0.003	36.5

Heat stroke (n = 150). Ordinary least squares path analysis with ln-transformed, batch-adjusted I-FABP (exposure) and LPS (mediator); effects are expressed per 1-unit increase in ln I-FABP and in SOFA points. Covariates: age, core temperature and onset-to-cooling delay. Bootstrap 5,000 resamples; a, b, c′, c: percentile 95% CI; indirect effect: bias-corrected and accelerated 95% CI; bootstrap two-sided p. Estimates describe statistical mediation in observational data and do not establish causation.

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Table 4. Discrimination of grade 3 heat stroke and incremental value of I-FABP, LPS, HSP70 and the mismatch index over clinical comparators

Predictor or model	AUC (95% CI)	Cut-off; sensitivity / specificity, %	AUC*	ΔAUC (95% CI), DeLong	p DeLong	p bootstrap	LR test p
Single markers (batch-adjusted concentrations, original units)							
Core temperature (°C)	0.57 (0.47–0.66)	≥ 41.0 °C; 71 / 48	—	—	—	—	—
Lactate (mmol/L)	0.75 (0.66–0.82)	≥ 1.9 mmol/L; 91 / 48	—	—	—	—	—
Procalcitonin (ng/mL)	0.83 (0.75–0.89)	≥ 0.95 ng/mL; 83 / 74	—	—	—	—	—
CRP (mg/L)	0.75 (0.66–0.82)	≥ 30.2 mg/L; 71 / 66	—	—	—	—	—
I-FABP (pg/mL)	0.78 (0.70–0.85)	≥ 2,320 pg/mL; 69 / 76	—	—	—	—	—
LPS (EU/mL)	0.78 (0.70–0.85)	≥ 0.303 EU/mL; 88 / 61	—	—	—	—	—
HSP70 (ng/mL), low values	0.76 (0.68–0.83)	≤ 6.75 ng/mL; 95 / 46	—	—	—	—	—
GCMI (z-units)	0.81 (0.74–0.87)	≥ 0.44; 78 / 72	—	—	—	—	—
Multivariable models (nested)							
M0: clinical (age, core temperature, cooling delay)	0.69 (0.59–0.77)	—	0.67	—	—	—	—
M1: M0 + I-FABP + LPS (vs M0)	0.82 (0.74–0.88)	—	0.80	0.13 (0.05–0.21)	0.001	0.032	<0.001
M2: M1 + HSP70 (vs M1)	0.85 (0.78–0.90)	—	0.82	0.03 (−0.01–0.06)	0.149	0.301	<0.001
M3: M0 + GCMI (composite) (vs M1)	0.84 (0.77–0.89)	—	0.83	0.02 (0.00–0.04)	0.094	0.093	—
Stronger clinical comparators (added after the primary analysis)							
M0L: M0 + lactate	0.78 (0.70–0.85)	—	0.76	—	—	—	—
M1L: M0L + I-FABP + LPS (vs M0L)	0.85 (0.78–0.90)	—	0.83	0.07 (0.02–0.12)	0.009	0.154	<0.001
M0P: M0L + procalcitonin + CRP	0.87 (0.80–0.92)	—	0.84	—	—	—	—
M1P: M0P + I-FABP + LPS (vs M0P)	0.87 (0.80–0.92)	—	0.84	0.00 (−0.01–0.02)	0.637	0.770	0.403

Grade 3 (n = 58) vs grades 1–2 (n = 92), heat stroke cohort. AUC with DeLong 95% CI; cut-off by Youden index (sample-derived, descriptive). Optimism-corrected AUC: Harrell bootstrap (1,000 resamples). ΔAUC: DeLong test of correlated AUCs; p bootstrap: out-of-bag paired bootstrap (2,000 resamples), two-sided; LR test: likelihood-ratio test of nested logistic models. M2 and M3 are compared with M1; M3 replaces the three marker terms by their composite and is not nested in M1 (no LR test). Clinical covariates: age, core temperature, onset-to-cooling delay; lactate, procalcitonin and CRP entered as standardized values. Admission SOFA defines grade 3 and is not shown. GCMI = z[ln I-FABP] + z[ln LPS] − z[ln HSP70], z-scored within the heat stroke cohort (constants in Methods). AUC: area under the ROC curve; CRP: C-reactive protein; SOFA: Sequential Organ Failure Assessment. *: Optimism-corrected AUC.

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Table 5: Adjusted associations of cooling delay, core temperature, age, and biomarkers with severity grade

Predictor	Grade 3 vs 1–2 aOR (95% CI), M2	Grade 3 vs 1–2 aOR (95% CI), M3	Ordinal grade aOR (95% CI), M2	VIF (M2)
Onset-to-cooling delay (per 1 h)	0.91 (0.76–1.09)	0.89 (0.75–1.05)	0.91 (0.78–1.05)	1.98
Core temperature (per 1 °C)	4.03 (1.30–12.48)*	2.55 (1.04–6.28)*	2.67 (1.11–6.42)*	1.60
Age (per 10 years)	1.30 (0.99–1.72)	1.31 (1.00–1.73)*	1.26 (1.00–1.58)	1.71
I-FABP (per 1 SD of ln value)	1.75 (0.83–3.69)	—	1.86 (0.98–3.54)	3.13
LPS (per 1 SD of ln value)	1.48 (0.66–3.32)	—	1.53 (0.79–2.97)	3.67
HSP70 (per 1 SD of ln value)	0.31 (0.15–0.64)**	—	0.38 (0.21–0.68)**	2.92
GCMI (per 1 SD)	—	6.69 (3.29–13.60)***	—	—

Heat stroke cohort (n = 150). Models M2 and M3 as in Table 4: binary logistic regression; ordinal grade: proportional-odds logistic regression (Brant test p = 0.957). aOR: adjusted odds ratio; VIF: variance inflation factor. Hosmer–Lemeshow p: M2 = 0.070, M3 = 0.784. *p < 0.05, **p < 0.01, ***p < 0.001; — = term not in model. The association of cooling delay with I-FABP and LPS is given in Table S1.

Table 6: Biomarker kinetics over 24 hours by severity grade and 28-day mortality in heat stroke

Biomarker	Window	Grade 1	Grade 2	Grade 3	Survivors	Non-survivors	p
I-FABP	0–6 h	0.61 (0.39–0.78)	0.59 (0.45–0.83)	0.89 (0.56–1.32)	0.62 (0.45–0.96)	0.83 (0.58–1.19)	0.024
	0–24 h	0.14 (0.09–0.26)	0.20 (0.13–0.34)	0.48 (0.24–0.82)	0.22 (0.12–0.41)	0.61 (0.21–1.02)	<0.001
LPS	0–6 h	1.03 (0.71–1.47)	1.24 (0.85–1.66)	1.74 (0.98–2.45)	1.24 (0.86–1.81)	1.88 (1.00–2.36)	0.019
	0–24 h	0.37 (0.19–0.65)	0.65 (0.36–0.88)	0.80 (0.47–1.33)	0.48 (0.29–0.87)	1.23 (0.63–1.73)	<0.001
HSP70	0–6 h	1.56 (1.12–2.26)	1.29 (0.89–1.96)	1.15 (0.76–1.61)	1.34 (0.89–2.02)	1.20 (0.88–1.62)	0.214
	0–24 h	2.32 (1.38–3.29)	2.04 (1.41–2.94)	1.26 (0.76–1.86)	2.01 (1.35–3.04)	1.08 (0.54–1.53)	<0.001

Fold change relative to admission (T/T0), median (interquartile range); > 1 indicates a rise and < 1 clearance. p: survivors vs non-survivors, Mann–Whitney U test. For all three markers, the Jonckheere–Terpstra trend across grades 1–3 and the likelihood-ratio tests of the grade × time and mortality × time interactions (linear mixed models of batch-adjusted ln concentration with a random intercept per patient, adjusted for age; measurements at T0, T6 and T24 in heat stroke only) gave p < 0.001. Five patients who died within 24 h have no T24 sample; they contribute T0 and T6 observations to the mixed models and are handled by sensitivity analyses (Table S1).

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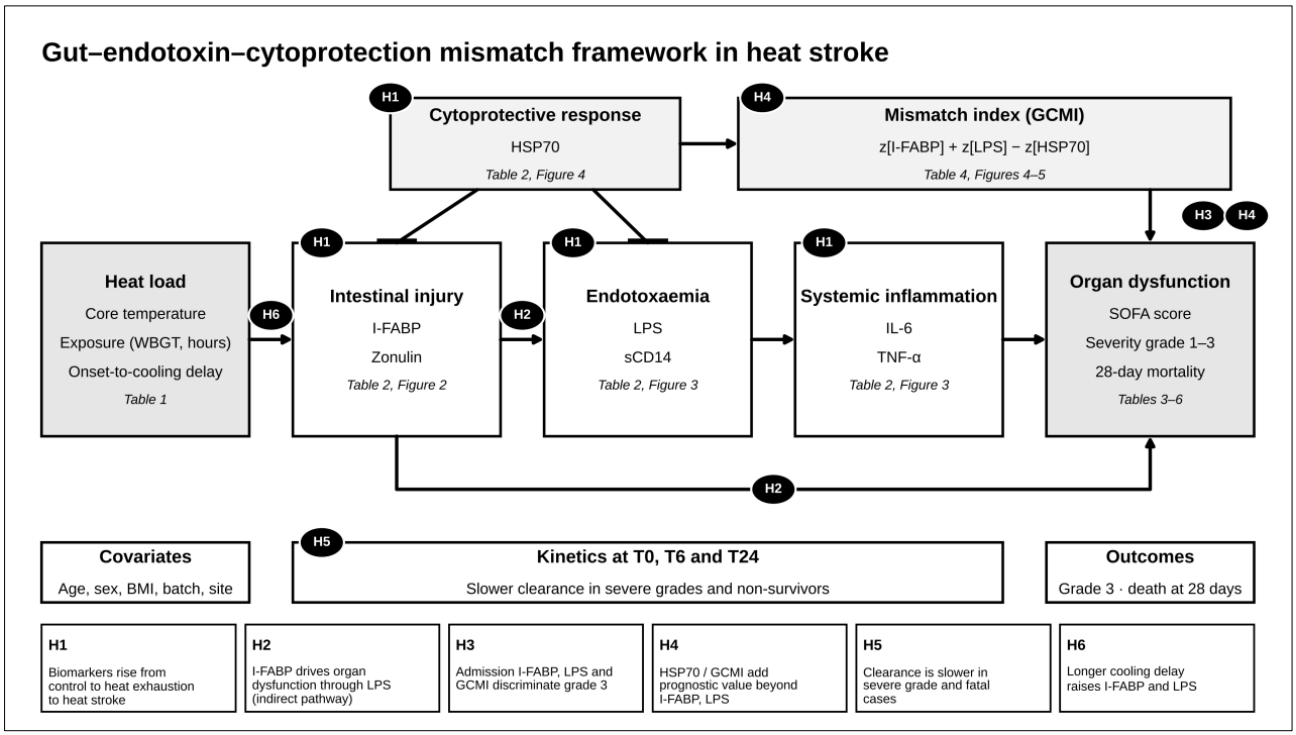


Figure 1: Gut–endotoxin–cytoprotection mismatch framework mapped to the study hypotheses (H1–H6). Boxes show study constructs and where they are analyzed; badges give the hypothesis tested. Arrows: assumed direction of effect; flat-ended lines: inhibitory (protective) influence. BMI: body mass index; GCMI: gut–cytoprotection mismatch index; HSP70: heat shock protein 70; I-FABP: intestinal fatty acid-binding protein; LPS: lipopolysaccharide; SOFA: Sequential Organ Failure Assessment; WBGT: wet-bulb globe temperature.

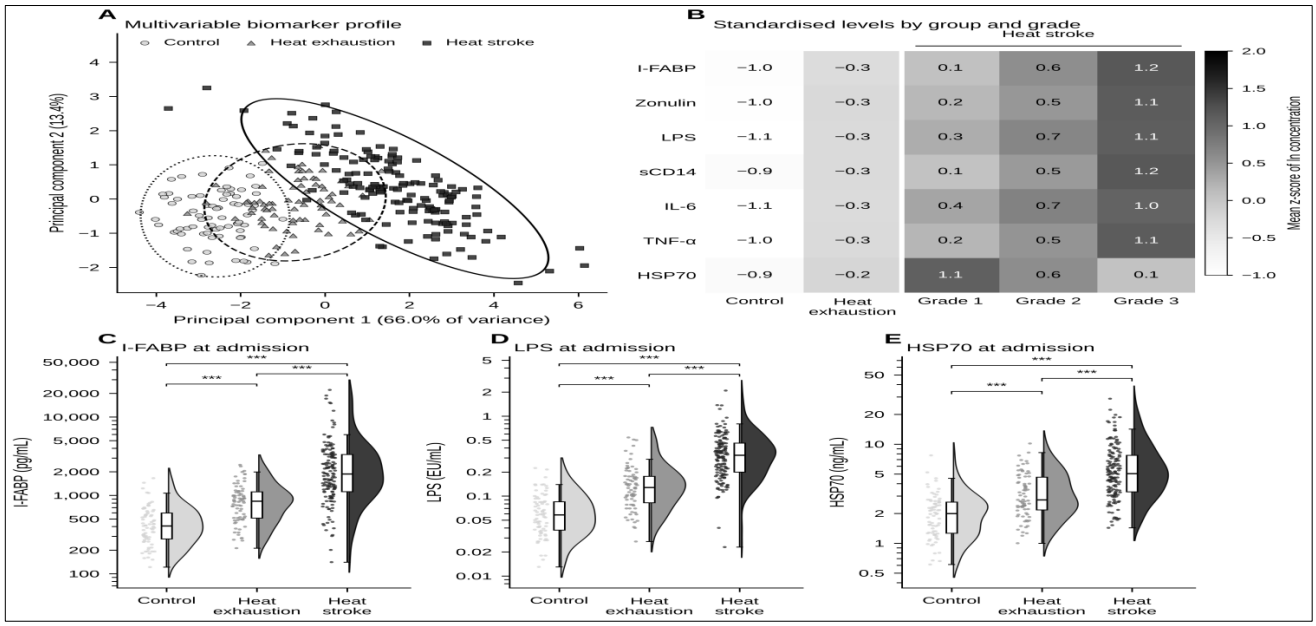


Figure 2: Biomarker landscape across control, heat exhaustion and heat stroke groups and severity grades. A: principal components of seven standardized, batch-adjusted ln biomarkers (ellipses: 95% data ellipses). B: mean z-scores of ln concentration by group and heat stroke grade. C–E: half-violin, box (median, interquartile range) and individual values on a log scale; brackets: Dunn test, Holm-adjusted p (***p < 0.001). I-FABP: intestinal fatty acid-binding protein; LPS: lipopolysaccharide; HSP70: heat shock protein 70.

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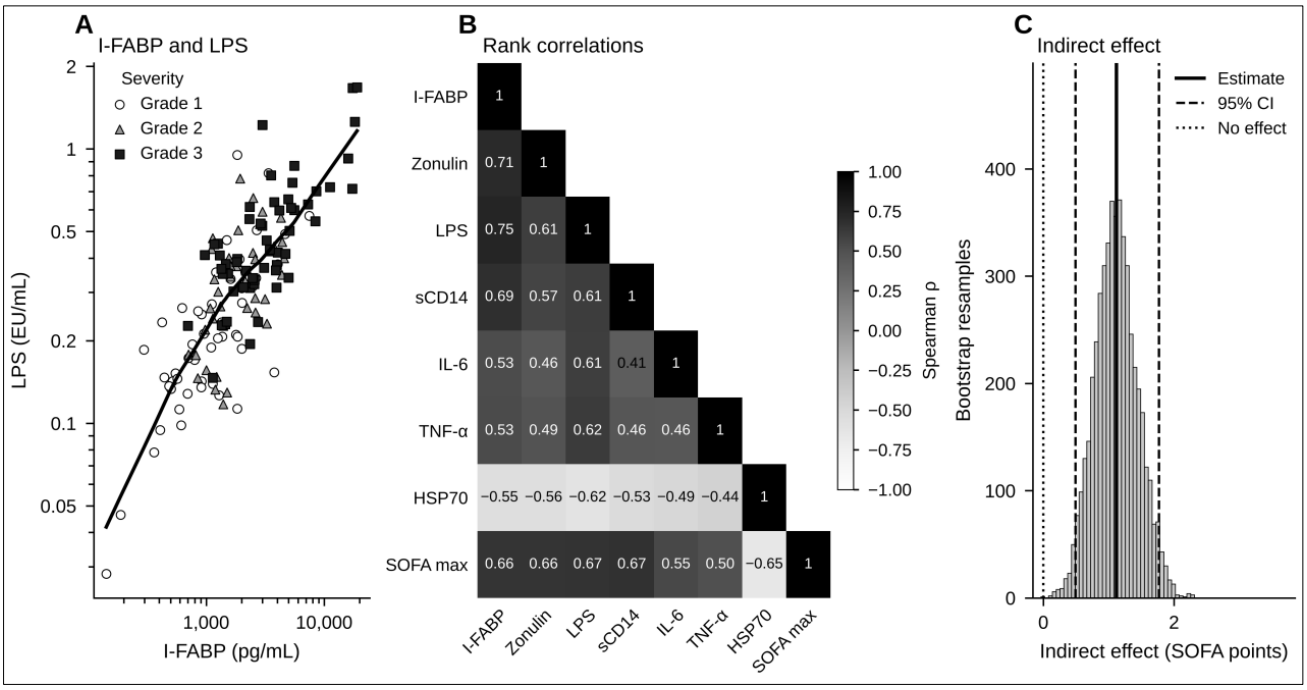


Figure 3: Coupling between intestinal injury, endotoxemia and organ dysfunction in heat stroke. A: batch-adjusted concentrations with LOWESS smoother (log–log scale). B: Spearman correlation matrix (SOFA max, maximum SOFA score within 72 h). C: 5,000 bootstrap resamples of the indirect effect I-FABP → LPS → maximum SOFA (solid line, estimate; dashed lines, BCa 95% CI).

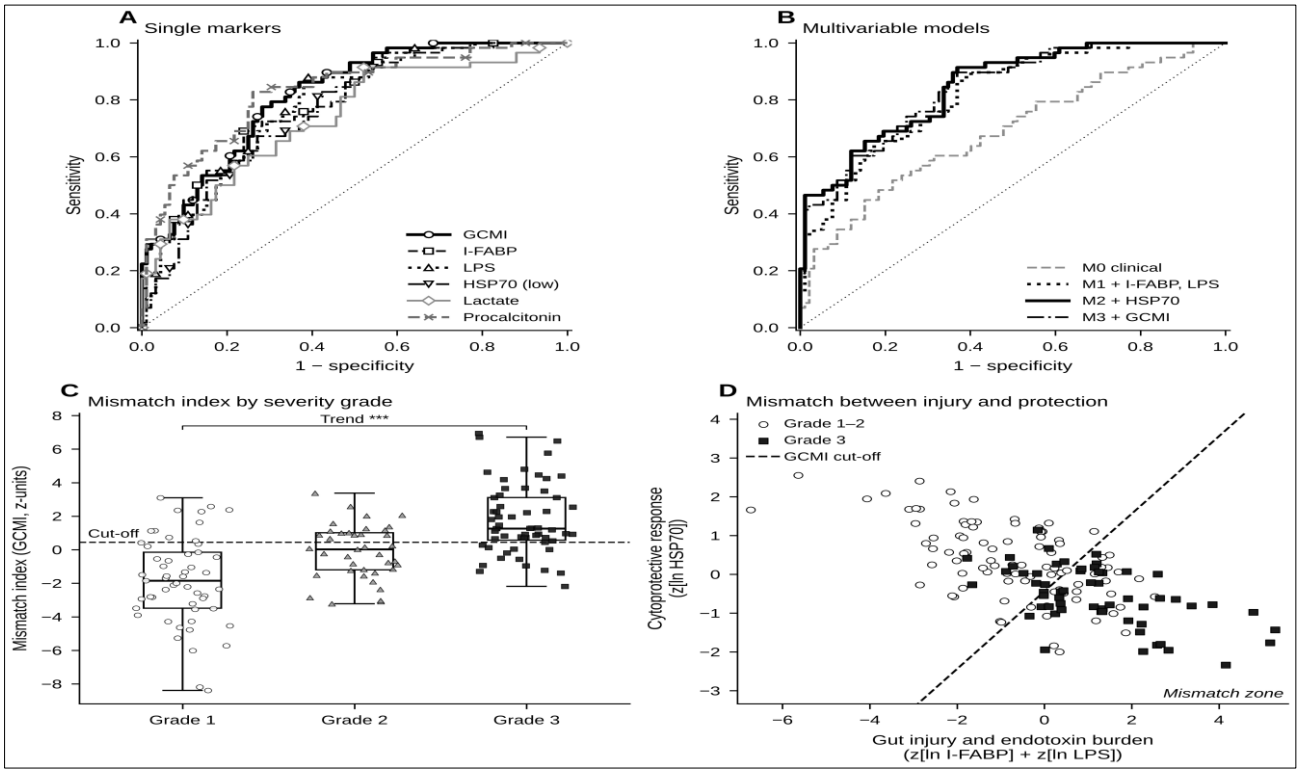


Figure 4: A: Discrimination of grade 3 heat stroke and the injury–protection mismatch signature. B: receiver operating characteristic curves for grade 3 vs. grades 1–2 (models M0–M3 as in Table 4; apparent). C: GCMI by severity grade (box: median, interquartile range; bracket: Jonckheere–Terpstra trend test, ***p < 0.001; dashed line: Youden cut-off). D: dashed line is the GCMI cut-off; the lower-right region combines high injury with low HSP70 (mismatch). GCMI: gut–cytoprotection mismatch index.

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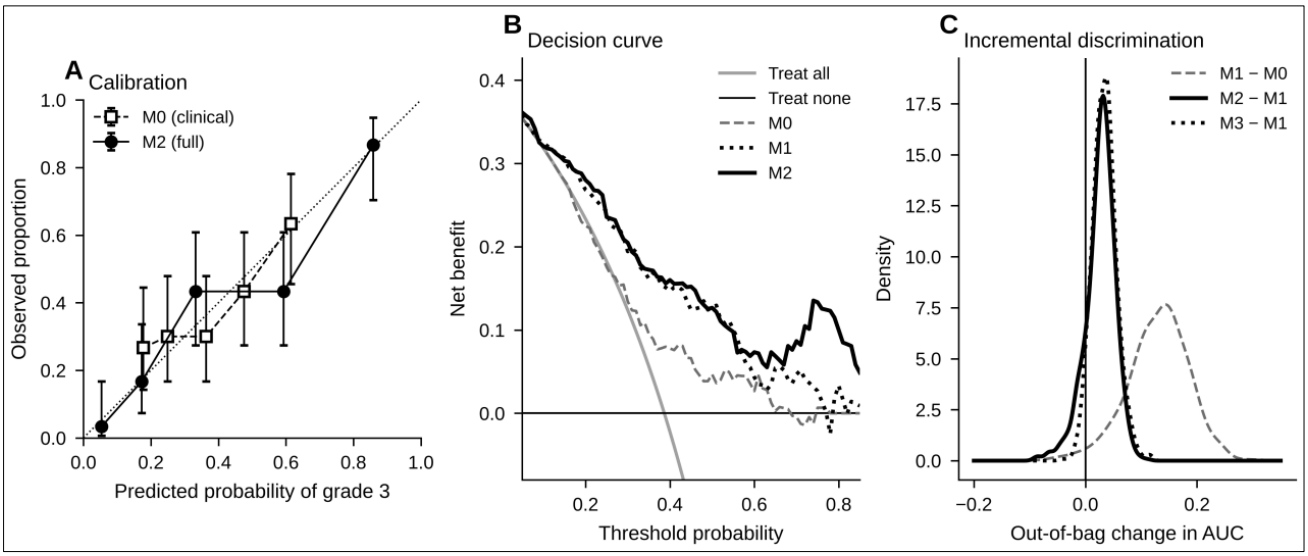


Figure 5: Calibration, clinical utility, and incremental discrimination of the biomarker model. Predictions from repeated 10-fold cross-validation (20 repeats). A: observed proportion by quintile of predicted risk (95% Wilson CI). B: net benefit across threshold probabilities. C: change in AUC in out-of-bag samples over 1,000 bootstrap resamples. M0, clinical model; M1, + I-FABP and LPS; M2, + HSP70; M3, M0 + GCMI.

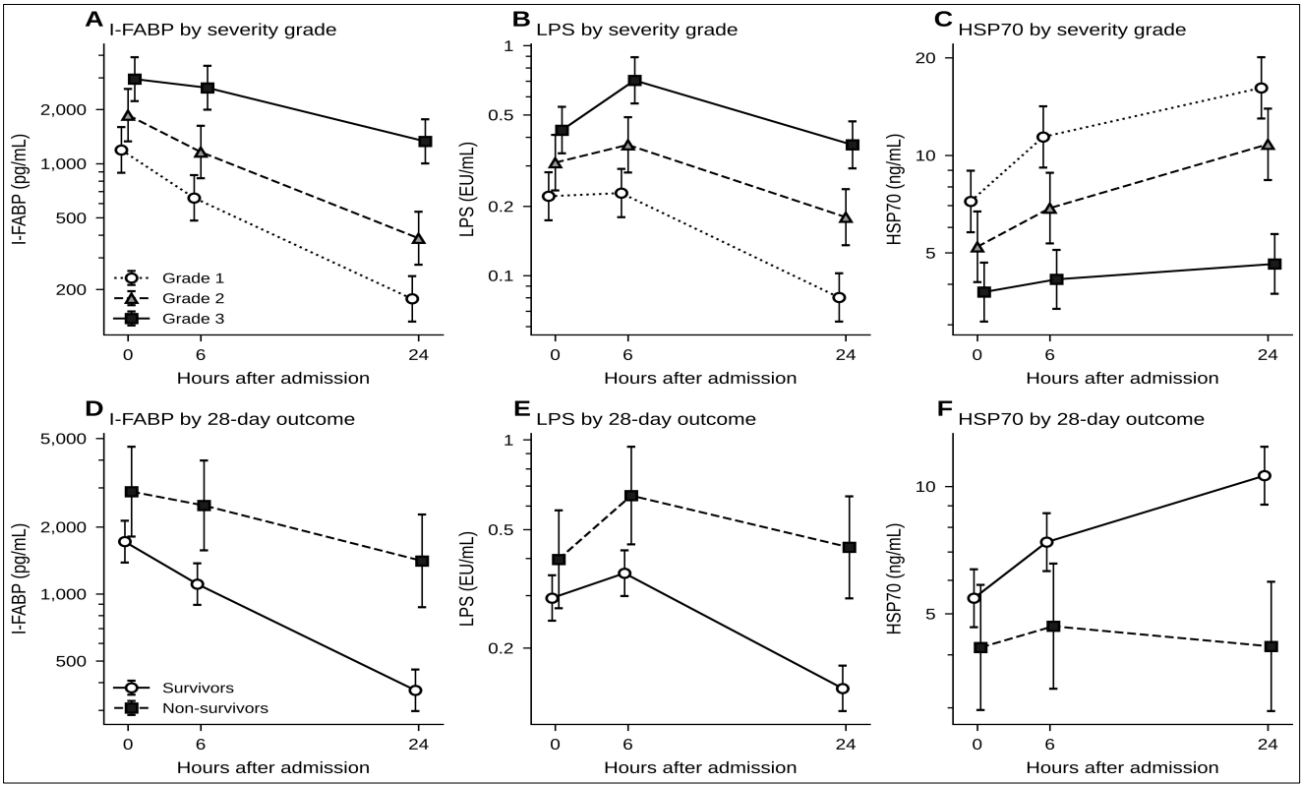


Figure 6: Biomarker kinetics over 24 hours by severity grade and 28-day outcome. Estimated geometric means (95% CI) from linear mixed models of batch-adjusted ln concentration (random intercept; time × severity grade or time × outcome; adjusted for age), back-transformed; points are offset horizontally for clarity. Five patients who died before 24 h contribute T0 and T6 values only.

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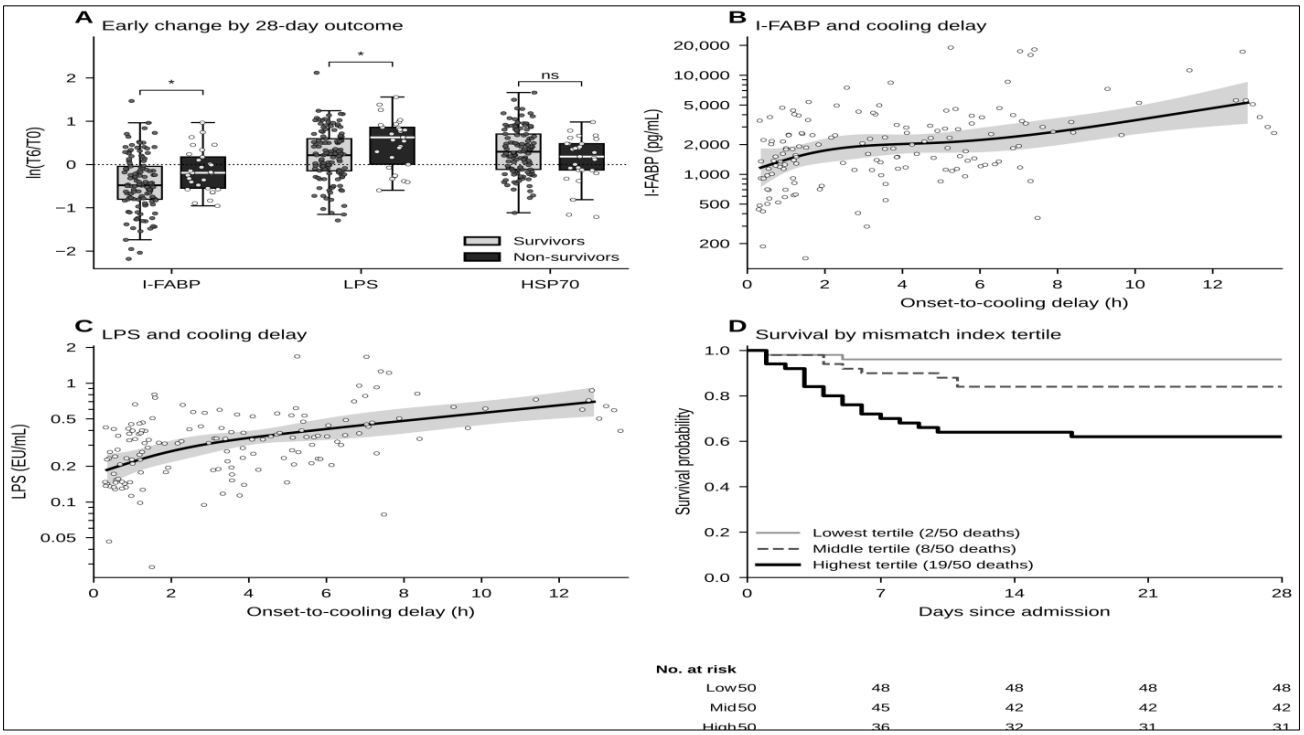


Figure 7: Early biomarker clearance, cooling delay and survival by mismatch index tertile. A: ln fold change from admission to 6 h (Mann–Whitney U test: *p < 0.05; ns: not significant). B, C: restricted cubic spline fit (4 knots) with 95% CI (HC3) adjusted for core temperature and age; circles are patients. D: Kaplan–Meier curves by tertile of GCMI (survivors censored at day 28; log-rank $\chi^2 = 20.0$, $p < 0.001$; numbers at risk are shown below the panel).

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8. Appendix

Supplementary Table 1: Sensitivity and robustness analyses of the primary findings

Analysis	Estimate	95% CI	p
Mediation and model sensitivity			
Indirect effect (I-FABP → LPS → maximum SOFA), + heat stroke subtype	1.11	0.50 to 1.77	<0.001
Indirect effect, unadjusted ln values with explicit ELISA batch terms	1.07	0.49 to 1.77	<0.001
Indirect effect, negative-binomial outcome model (log rate ratio)	0.162	0.080 to 0.252	<0.001
AUC of M2, + heat stroke subtype (LR p for biomarkers beyond M0 + subtype)	0.85	0.78 to 0.90	<0.001
AUC of M2, with explicit ELISA batch terms (LR p for biomarkers beyond M0 + batch)	0.86	0.79 to 0.91	<0.001
Leave-one-site-out AUC of M2, Site 1 held out (n = 66; grade 3 = 26)	0.80	0.67 to 0.89	—
Leave-one-site-out AUC of M2, Site 2 held out (n = 40; grade 3 = 14)	0.80	0.62 to 0.91	—
Leave-one-site-out AUC of M2, Site 3 held out (n = 44; grade 3 = 18)	0.82	0.65 to 0.91	—
ΔAIC, M3 minus M1 (negative favors the composite)	−11.0	—	—
Sampling delay and discrimination of grade 3			
AUC of M0 + sampling delay / M1 + sampling delay	0.68 / 0.82	0.59 to 0.77 / 0.74 to 0.88	—
ΔAUC, M1 minus M0, out-of-bag paired bootstrap (2,000 resamples)	0.13	0.01 to 0.23	0.032
ΔAUC, M2 minus M1, bootstrap	0.03	−0.04 to 0.08	0.301
ΔAUC, M3 minus M1, bootstrap	0.04	0.00 to 0.09	0.093
ΔAUC, M1L minus M0L (lactate comparator), bootstrap	0.06	−0.03 to 0.14	0.154
ΔAUC, M1P minus M0P (lactate, procalcitonin, CRP comparator), bootstrap	−0.01	−0.06 to 0.02	0.770
ΔAUC, M1 minus M0, both with sampling delay, bootstrap	0.14	0.02 to 0.25	0.024
Sampling delay, odds ratio for grade 3 per 10 min (M1 + delay)	0.90	0.68 to 1.20	0.475
Sampling delay, Spearman ρ with ln I-FABP; LPS; HSP70	−0.03; 0.04; 0.02	—	0.738; 0.608; 0.849
Indirect effect (maximum SOFA), + sampling delay	1.15	0.47 to 1.81	<0.001
Indirect effect (maximum SOFA), + subtype, comorbidity, and site	1.10	0.50 to 1.79	<0.001
ELISA batch against site; group; admission month, χ^2 test	—	—	0.32; 0.55; 0.057
Cooling delay (ln I-FABP; ln LPS), geometric mean ratio			
Primary model (age, core temperature), per 1 h	1.11; 1.10	1.06 to 1.16; 1.06 to 1.15	<0.001; <0.001
+ sampling delay, per 1 h	1.11; 1.10	1.06 to 1.16; 1.06 to 1.15	<0.001; <0.001
+ heat stroke subtype, per 1 h	1.16; 1.13	1.10 to 1.23; 1.08 to 1.18	<0.001; <0.001
+ exposure before onset, per 1 h	1.12; 1.11	1.06 to 1.19; 1.06 to 1.16	<0.001; <0.001
Door-to-cooling time instead, per 10 min	1.13; 1.10	0.96 to 1.33; 0.96 to 1.26	0.157; 0.176
Non-linearity, restricted cubic spline (4 knots), F test	F = 0.66; 1.14	—	0.519; 0.322
Clearance, non-survivors minus survivors (ln I-FABP T24/T0; ln LPS T24/T0), Hodges–Lehmann difference			
Complete cases	0.86; 0.79	0.38 to 1.37; 0.41 to 1.16	<0.001; <0.001
Last observation carried forward (T6 for T24)	0.87; 0.86	0.45 to 1.27; 0.51 to 1.15	<0.001; <0.001
Best case (median survivor clearance imputed)	0.66; 0.59	0.28 to 1.12; 0.28 to 1.01	0.002; <0.001
Values below the quantification limit set to the limit (T24; I-FABP; LPS)	0.80; 0.77	0.33 to 1.31; 0.41 to 1.16	<0.001; <0.001
Adjusted clearance ratio (fold change T24/T0), age, ln creatinine, ln baseline; n = 145			
Non-survivors vs survivors: I-FABP; LPS; HSP70	1.27; 1.36; 0.69	0.81 to 2.00; 0.90 to 2.05; 0.50 to 0.95	0.296; 0.142; 0.023
Grade 3 vs grades 1–2: I-FABP; LPS; HSP70	1.52; 1.09; 0.82	1.01 to 2.29; 0.78 to 1.54; 0.64 to 1.05	0.045; 0.609; 0.113
Prognostic endpoints (heat stroke, n = 150)			
AUC for 28-day death: SOFA; procalcitonin; GCMI	0.85; 0.86; 0.76	0.76 to 0.91; 0.77 to 0.92; 0.65 to 0.85	—
Death: AUC of SOFA + age without / with GCMI; odds ratio of GCMI per SD	0.888 / 0.888; 0.89	0.38 to 2.08	0.795
Death: same with CRP in the base model; odds ratio of GCMI	0.892 / 0.892; 1.03	0.42 to 2.51	0.953
Death, SOFA recomputed from its components: AUC of SOFA + age without / with GCMI; odds ratio of GCMI	0.889 / 0.890; 0.86	0.37 to 2.01	0.733

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Analysis	Estimate	95% CI	p
Early deterioration (30 events): AUC of SOFA + age without / with GCMI; odds ratio	0.68 / 0.82; 5.4	2.4 to 12.0	<0.001
Early deterioration: ΔAUC with GCMI, DeLong; bootstrap p	0.14	0.04 to 0.24	0.007; 0.070
Early deterioration: same with CRP in the base model, AUC 0.83 / 0.85; odds ratio	3.4	1.5 to 8.1	0.004
ICU admission (80 events): AUC of SOFA + age without / with GCMI; odds ratio	0.889 / 0.892; 1.36	0.67 to 2.75	0.391

Note. Paired values are I-FABP; LPS (or I-FABP; LPS; HSP70 where stated). Percentile bootstrap 95% CI (BCa for the linear mediation model; fixed seed 20260101); bootstrap p is two-sided. AIC: Akaike information criterion; LR: likelihood ratio.

Supplementary Table 2: Immunoassay and LAL kit specifications and assay performance

Analyte	Sample	Samples, n; observed range	Manufacturer, catalog no.	LLOQ	ULOQ	Minimum predilution	Intra-assay CV	Inter-assay CV
I-FABP	Serum	595; 3.7–67,783 pg/mL	Hycult Biotech, HK406	47 pg/mL	3,000 pg/mL	1:23	n/a	n/a
Zonulin	Serum	300; 17.5–249.2 ng/mL	Immundiagnostik, K 5601	0.183 ng/mL	n/a	1:20	3.5–6.0%	7.7–8.3%
LPS	Plasma	595; 0.001–10.6 EU/mL	Thermo Scientific Pierce, A39552	0.01 EU/mL	1.0 EU/mL	1:11	n/a	n/a
sCD14	Serum	300; 920–8,140 ng/mL	R&D Systems, DC140	0.25 ng/mL	16 ng/mL	1:509	4.8–6.4%	4.8–7.4%
IL-6	Serum	300; 0.3–370.6 pg/mL	R&D Systems, HS600C	0.156 pg/mL	10 pg/mL	1:38	5.2%	8.1%
TNF-α	Serum	300; 1.0–103.9 pg/mL	R&D Systems, HSTA00E	0.156 pg/mL	10 pg/mL	1:11	1.9–2.2%	6.2–6.7%
HSP70	Serum	595; 0.54–130.3 ng/mL	Enzo Life Sciences, ADI-EKS-715	0.20 ng/mL	12.5 ng/mL	1:11	n/a	n/a

CV: coefficient of variation; LLOQ and ULOQ: lower and upper limits of quantification; n/a: not applicable or not available. LLOQ is the lowest calibration standard (zonulin: manufacturer limit of quantification; LPS: kit sensitivity). Minimum predilution is the dilution that brings the highest observed value within the ULOQ (zonulin: manufacturer standard serum dilution). Observed ranges are the reported concentrations at T0, T6 and T24 (sCD14 is given in ng/mL here and in µg/mL elsewhere). CVs are the manufacturers' published values, except IL-6, which are this study's own; CVs for I-FABP, LPS and HSP70 are not published and were not measured in-house. Thirteen values (11 I-FABP, 2 LPS; all at T6 or T24) were below the LLOQ and were analyzed as measured (Table S1). LPS spike recovery was 85–115%.

Supplementary Table 3: Data-quality, assumption, and confounding audit

Variable	Missing, n (%)	Skewness (raw)	Skewness (ln)	Shapiro p (residuals)	Levene p (groups)	ELISA batch p	Site p	VIF (M2)
I-FABP (pg/mL)	0 (0.0)	4.57	0.37	0.079	<0.001	0.123	0.840	3.13
Zonulin (ng/mL)	0 (0.0)	1.99	0.17	0.544	0.283	<0.001	0.771	—
LPS (EU/mL)	0 (0.0)	2.86	−0.17	0.050	0.965	0.024	0.727	3.67
sCD14 (µg/mL)	0 (0.0)	1.50	0.10	0.114	0.130	<0.001	0.455	—
IL-6 (pg/mL)	0 (0.0)	2.85	−0.11	<0.001	0.120	0.201	0.801	—
TNF-α (pg/mL)	0 (0.0)	2.82	0.17	0.888	0.005	0.002	0.563	—
HSP70 (ng/mL)	0 (0.0)	2.48	0.21	0.510	0.163	0.272	0.408	2.92
Age (years)	0 (0.0)	0.96	—	<0.001	<0.001	—	—	1.71
Body mass index (kg/m^2)	0 (0.0)	0.17	—	0.163	0.396	—	—	—
Core temperature (°C)	0 (0.0)	−0.25	—	<0.001	<0.001	—	—	1.60
Onset-to-cooling time (min)	75 (25.0)	1.59	—	<0.001	<0.001	—	—	1.98
Sampling delay at T0 (min)	0 (0.0)	0.44	—	<0.001	<0.001	—	—	—
Maximum SOFA, 0–72 h	75 (25.0)	0.67	—	<0.001	<0.001	—	—	—

Biomarker rows: residual Shapiro–Wilk test from the ANCOVA of ln values; Levene (median-centered) test of equal variance across the three groups on the ln scale; ELISA batch and site: Wald F test of batch (5 df) and site (2 df) terms in the ANCOVA (HC3). Other rows: Shapiro–Wilk and Levene tests on the raw scale. Group distribution across ELISA batches p = 0.548; across sites p = 0.863. VIF: variance inflation factor in model M2 (Table 4). T6 and T24 samples exist in heat stroke only (five early deaths have no T24 sample); cooling time and SOFA were not collected in controls. Skewed markers were ln-transformed; no observations were removed.

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