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Biomarkers of heatstroke induced organ injury and repair

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Abstract

Classic and exertional heatstroke cause acute injury and damage across numerous organ systems. Moreover, heatstroke survivors may sustain long-term neurologic, cardiovascular, and renal complications with a persistent risk of death. In this context, biomarkers, defined as biological samples obtained from heatstroke patients, are needed to detect early organ injury, and predict outcomes to develop novel organ preservation therapeutic strategies. This narrative review provides preliminary insights that will guide the development and future utilization of these biomarkers. To this end, we have identified numerous biomarkers of widespread heatstroke-associated cellular injury, tissue damage and repair (extracellular heat shock proteins 72 and 60, high mobility group box protein 1, histone H3, and interleukin-1α), and other organ-specific biomarkers including those related to the cardiovascular system (cardiac troponin I, endothelium-derived factors, circulation endothelial cells, adhesion molecules, thrombomodulin, and von Willebrand factor antigen), the kidneys (plasma and urinary neutrophil gelatinase-associated lipocalin), the intestines (intestinal fatty acid-binding protein 2), the brain (serum s100β and neuron-specific enolase) and skeletal muscle (creatine kinase, myoglobin). No specific biomarkers have been identified so far for liver or lung injury in heatstroke. Before translating the identified biomarkers into clinical practice, additional preclinical and clinical prospective studies are required to further understand their clinical utility, particularly for the biomarkers related to long-term post-heatstroke health outcomes.

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Keywords

Exertional heatstroke; classic heatstroke; biomarkers; organ systems; multiorgan failure; hyperthermia

Introduction

With global temperatures rising, heatwaves have become more frequent, intense, and longer in duration (IPCC, 2021), thereby exposing a greater proportion of the population to the deadly risk of extreme heat (Mora *et al.*, 2017). Heat exposure elevates the risk of developing heat illness, which describes a continuum of pathologies ranging from relatively mild conditions associated with alterations in cardiovascular functioning (e.g., heat syncope, heat exhaustion) to potentially lethal heatstroke (Armstrong *et al.*, 2007). Heatstroke is characterized by a rapidly rising core temperature (usually >40.1°C), central nervous system dysfunction, systemic inflammation, and organ system injury that can culminate in death (Bouchama *et al.*, 2022a). Heatstroke manifests in two distinct forms, classic heatstroke (CHS) and exertional heatstroke (EHS) (Bouchama *et al.*, 2022a). CHS typically occurs during resting exposures to hot and humid conditions, which limit the capacity for heat loss, and more frequently affects the very young, older adults, and populations with comorbidities (Bouchama *et al.*, 2022a). EHS more often occurs in younger, physically active, healthy adults, such as military personnel and competitive or recreational athletes, and is characterized by the inability to adequately dissipate enough heat sufficient to offset the heat load caused by physical exertion (i.e., elevations in metabolic heat production) (Bouchama *et al.*, 2022a). As a result, EHS can occur across a comparatively wide range of environmental conditions (Rae *et al.*, 2008; Stacey *et al.*, 2022). Therefore, the threat of heatstroke can impact the health and well-being of millions of people around the globe, and this risk will likely continue to increase in the coming decades (IPCC, 2021).

Heatstroke is diagnosed mainly through clinical signs and symptoms. Hallmarks of heatstroke include an elevated core temperature (often >40.1°C) and central nervous system alterations (e.g., confusion, irritability, altered consciousness) (Casa *et al.*, 2015; Bouchama *et al.*, 2022a) combined with a relevant patient history of exposure to hot and humid conditions and/or strenuous physical exertion (Leon & Bouchama, 2011). Notably, however, a core temperature less than 40.1°C should never rule out the diagnosis of heatstroke as the measurement of core temperature is often delayed, usually after the patient's removal from environmental heat and/or cessation of the exercise and arrival of paramedics. With early diagnosis and rapid cooling, the prognosis for heatstroke victims is generally good (Demartini *et al.*, 2015). In contrast, the risk of prolonged morbidity and mortality increases when heatstroke diagnosis and treatment are delayed, resulting in tissue damage and multiorgan dysfunction/failure, and eventually death. This evolution has been attributed to heat cytotoxicity, excessive systemic inflammation, and hypercoagulability (Leon & Bouchama, 2011; Casa *et al.*, 2012; Bouchama *et al.*, 2022a). The systemic nature of heatstroke pathology is exemplified in an analysis of 2,529 EHS episodes occurring in military personnel from 2008 to 2014, demonstrating abnormal clinical indices of liver, renal and hematological function and muscle damage that peak 0–4 days following diagnosis and

persist for up to 16 days post injury (Ward *et al.*, 2020). These findings corroborate animal literature demonstrating widespread damage across numerous organ systems following CHS (Leon *et al.*, 2006b; Roberts *et al.*, 2008a) and EHS (King *et al.*, 2015). Moreover, observational evidence indicates that a single episode of heatstroke could have deleterious long-term all-cause mortality (Wallace *et al.*, 2007), neurologic (Dematte *et al.*, 1998; Argaud *et al.*, 2007; Yang *et al.*, 2017), cardiovascular (Wallace *et al.*, 2007; Wang *et al.*, 2019) and renal (Wang *et al.*, 2019; Wu *et al.*, 2021) complications with a continuous risk of death (Bouchama *et al.*, 2022a). These observations have raised the need to develop tools that aid in detecting tissue and organ injury, monitoring recovery (or repair failure), and predicting long-term complications following heatstroke (Figure 1). Therefore, this narrative review provides an up-to-date overview of novel biological markers (biomarkers) that could help identify the patients at risk of organ injury or long-term complications following heatstroke. Our goal is not to provide recommendations for the clinical deployment of biomarkers because the literature base remains too underdeveloped for this to be reasonably accomplished. Rather, we will provide preliminary insights on biomarkers that could aid the prediction of short-term health outcomes in heatstroke and ultimately help develop novel preservation strategies to reduce organ damage and potentially prevent long-term sequela (Figure 2).

For this review, a *biomarker* is defined as a measurable substance derived from a bodily fluid that does not require assessment of clinical signs and symptoms. Notably, this definition purposely excludes standard clinical and biochemical tests that are helpful for patient care but are not specific to heatstroke. Furthermore, in this review, the term heatstroke will refer to observations that are consistent between CHS and EHS. At the same time, CHS and EHS will be used when referring to specific classic or exertional heatstroke observations or pathophysiology. Moreover, we will focus this review on tissues, organs and organ systems impacted by heatstroke. In the interest of conciseness, we will not provide an in-depth overview of the etiology of heatstroke, except where it is relevant to understanding injury biomarkers. Likewise, we will not be addressing risk predictive or diagnostic biomarkers of heatstroke, but we invite the interested reader to explore studies on these topics (e.g., (Lu *et al.*, 2004a; Alele *et al.*, 2021; Stacey *et al.*, 2022)).

Generalized cellular injury and tissue damage

Heatstroke is associated with a complex innate immune response characterized by neutrophil and complement protein activation, production of pro- and anti- inflammatory cytokines, chemokines, and acute phase proteins (Bouchama *et al.*, 2022b). The activation of the immune response has been attributed to endotoxin leaking from the heat-damaged gastrointestinal tract (Hall *et al.*, 1999) and danger signal molecules or alarmins released in the circulation from damaged or dying cells (Gallucci & Matzinger, 2001). Alarmins, also known as damage-associated molecular patterns, play a crucial role in removing necrotic tissue debris and other repair mechanisms (Chen & Nuñez, 2010). Alarmins have been implicated in the excessive and sustained inflammatory response leading to the production of reactive oxygen species and proteolytic enzymes that cause further tissue damage and the progression to multiorgan failure (Chen & Nuñez, 2010). Several alarmins have been identified in heatstroke patients (Huisse *et al.*, 2008; Ruell *et al.*, 2014) and preclinical

models (Dehbi *et al.*, 2010; Bruchim *et al.*, 2015). These include extracellular heat shock protein (eHSP)72 (Huisse *et al.*, 2008; Dehbi *et al.*, 2010; Ruell *et al.*, 2014; Bruchim *et al.*, 2015) and eHSP60 (Huisse *et al.*, 2008; Dehbi *et al.*, 2010), high mobility group box protein 1 (HMGB1) (Tong *et al.*, 2011b; Dehbi *et al.*, 2012), histone H3 (Bruchim *et al.*, 2017; Li *et al.*, 2021), and interleukin-1α (IL-1α) (Bouchama *et al.*, 1991).

Heat shock proteins are a family of proteins produced by cells when exposed to stressful conditions, including extreme heat. Heat shock proteins are produced intracellularly and function as chaperones to prevent heat-induced protein misfolding or aggregation (Kultz, 2005). eHSPs do not exert a chaperone function but act as cytokines engaging pattern-recognition receptors, such as toll-like receptors, to elicit an inflammatory response (Chen & Nuñez, 2010). eHSP72 is released in a sustained manner for up to 72 hours in non-human primate models of severe heatstroke and was associated with multiorgan damage and death, suggesting a role as a prognostic biomarker (Dehbi *et al.*, 2010). Moreover, high eHSP70 and eHSP60 were documented in the plasma of heatstroke patients, and the former correlated with plasma interleukin-8 concentrations (Huisse *et al.*, 2008). HMGB1, histone, and IL-1α are chromatin-associated nuclear proteins that are released by injured or dying cells (Cohen *et al.*, 2010). HMGB1 was demonstrated as an early mediator of inflammation, tissue injury and lethality in rodent model of heatstroke (Dehbi *et al.*, 2012), and plasma HMGB1 is associated with heatstroke severity and mortality (Huisse *et al.*, 2008). Likewise, histone H3 was linked to coagulopathy, and heatstroke severity and lethality (Bruchim *et al.*, 2017; Li *et al.*, 2021). Finally, plasma concentrations of IL-1α were increased in small cohort of CHS patients (Bouchama *et al.*, 1991). Notably, IL-1α functions intracellularly as transcription factor in healthy cells, but upon release in circulation behave as proinflammatory cytokine and contributes to tissue remodeling and repair (Cohen *et al.*, 2010). Overall, alarmins are promising biomarkers of heatstroke-associated tissue damage and repair that deserve further validation studies.

Cardiovascular system

Heart

Elevations in body temperature have profound effects on the cardiovascular system to support the increases in skin blood flow necessary to promote heat loss (Crandall & Wilson, 2014). The heart responds by increasing cardiac output, mediated mainly through increases in heart rate and the maintenance of stroke volume, which is made possible by increases in cardiac contractility (Crandall & Wilson, 2014). Given these cardiovascular effects, particularly when paired with the profuse hematological and vascular effects during and following heatstroke, it is not surprising that a single episode of heatstroke increases the risk of all-cause mortality by 40% (Wallace *et al.*, 2007). More specifically, patients with EHS display a higher incidence of cardiovascular disease, myocardial infarction, ischemic heart disease, and acute ischemic stroke as early as 14 years following the initial heatstroke event (Wallace *et al.*, 2007; Wang *et al.*, 2019).

Evidence of potential myocardial injury during heatstroke was preliminarily identified in a case report published in 2006 of a dog presenting with suspected EHS arnd elevations in cardiac troponin I (Mellor *et al.*, 2006), which increases when myocardial cells become

damaged. These findings were corroborated in a subsequent case report in humans (Whiticar *et al.*, 2008), including one case report reporting evidence of cardiomyopathy following heatstroke (Chen *et al.*, 2012). Indeed, EHS has recently been shown to provoke myocardial metabolic alterations consistent with the presence of myocardial injury that persists for up to two weeks following heatstroke (Laitano *et al.*, 2020) and marked immunosuppression that last at least one month but likely longer (Murray *et al.*, 2021). Interestingly, these observations were specific to female mice (Laitano *et al.*, 2020; Murray *et al.*, 2021), which was attributed to greater heat tolerance resulting in a higher net heat load than male mice before EHS occurred. Given the consistency of cardiac ramifications during and after heatstroke, a cardiac-specific biomarker likely has utility. To this end, there is a growing body of evidence supporting cardiac troponin I as a sensitive biomarker of myocardial injury during and following heatstroke. For instance, and consistent with the aforementioned case reports (Mellor *et al.*, 2006; Whiticar *et al.*, 2008), graded increases in circulating cardiac troponin I were observed with increased severity of heatstroke 24 hours following heatstroke in a rodent model of CHS (Quinn *et al.*, 2014). Moreover, a point-of-care assessment of cardiac troponin I predicted heatstroke severity (r^2=0.83) in a rodent model of CHS (Audet *et al.*, 2015). Finally, circulating cardiac troponin I may have longer-term prognostic utility. For example, higher levels of cardiac troponin I are associated with a higher death rate following emergency department admission during heatwaves (Hausfater *et al.*, 2010). Particularly relevant for EHS, is that cardiac troponin I can be elevated during prolonged exercise (e.g., marathon) (Shave *et al.*, 2007). Thus, the specificity of cardiac troponin I to heatstroke induced cardiac injury remains to be determined.

Blood vessels

Clinical and experimental studies suggest that changes in vascular endothelium and coagulation functions are characteristic features of heatstroke (Sohal *et al.*, 1968; Shieh *et al.*, 1995; Bouchama *et al.*, 1996b; Roberts *et al.*, 2008b). Moreover, functional alterations in these systems are also strongly associated with an increased risk of cardiovascular morbidity and mortality (Hadi *et al.*, 2005; Lowe & Rumley, 2014). Thus, it is not surprising that the risk of cardiovascular disease is elevated following a single heatstroke occurrence (Wang *et al.*, 2019). Postmortem studies in heatstroke patients revealed extensive endothelial damage and hemorrhagic thrombosis in most body organs (Malamud *et al.*, 1946a; Sohal *et al.*, 1968; Chao *et al.*, 1981). These findings were replicated and expanded in non-human primate models of heatstroke, demonstrating that endothelial cell, inflammation, and coagulation activation precede endothelial damage, thrombosis, and bleeding, which culminates in death (Sohal *et al.*, 1968; Roberts *et al.*, 2008b). Hence the capacity to detect early changes in endothelial function may have clinical utility (Bouchama *et al.*, 2005; Roberts *et al.*, 2008b). Several studies have investigated potential circulating biomarkers of endothelial dysfunction, including their link with heatstroke-induced organ damage, severity, and outcomes (Sohal *et al.*, 1968; Shieh *et al.*, 1995; Bouchama *et al.*, 1996b; Hammami *et al.*, 1998a; Alzeer *et al.*, 1999; Roberts *et al.*, 2008b). These include mediators of vascular tone, hemostasis, leucocyte trafficking, and endothelium barrier integrity.

Vascular tone—A series of elegant experimental studies by Hall, et al., demonstrated a central role of nitric oxide in the progression from heat stress to heatstroke (Hall *et al.*,

1994; Hall *et al.*, 2001). Indeed, hyperthermia stimulates nitric oxide synthesis and reactive oxygen species production in the rodent splanchnic circulation, resulting in intestinal barrier dysfunction, cardiovascular collapse, and heatstroke. An imbalance consisting of a marked release of endothelin-1 concomitant to reduced nitric oxide production in the liver sinusoid was also demonstrated in rodents (Zhang *et al.*, 2018). High levels of nitric oxide, assessed by nitrate and nitrite were identified in a small cohort of heatstroke patients that correlated with the severity of heatstroke on admission and mortality (Alzeer *et al.*, 1999). Endothelin-1, another endothelium-derived factor with dual properties including vasoconstrictive and vasodilating effects at higher and lower concentrations, was also detected in the splanchnic circulation of heatstroke patients although its clinical significance remains unknown (Ohlstein *et al.*, 1990; Bouchama *et al.*, 1996b). Taken together, these findings suggest that mediators of vascular tone are implicated in the pathogenesis of heatstroke and may warrant further evaluation as biomarkers of disease activity and survival.

Hemostasis—The coagulopathy of heatstroke progresses from the initial activation of coagulation and fibrinolysis followed by late fibrinolytic inhibition, resulting in a hypercoagulable state. Clinically, the patients with heatstroke display low platelet count, fibrinogen levels and increasing coagulation times, and D-dimer levels (al-Mashhadani *et al.*, 1994; Bouchama *et al.*, 1996a). However, except for D-dimer, these conventional coagulation parameters are not likely sensitive enough or specific to be used as biomarkers of organ injury in heatstroke. When assessed in research settings using sensitive assays such as thrombin-antithrombin and plasmin-antiplasmin complexes, one can detect early rise in thrombin and plasmin, with concomitant decrease of natural anticoagulant proteins (Protein C, S, and antithrombin III) before the alteration of coagulation tests used in clinical practice. Thrombin ($r=0.635$, $p=0.01$), plasmin ($r=0.577$, $p=0.02$), and D-dimer ($r=0.76$, $p=0.003$) correlate significantly with the magnitude of hyperthermia in CHS (Bouchama *et al.*, 1996a), whereas the levels of protein C and antithrombin III discriminate outcome severity in heatstroke, with lower concentrations of these biomarkers being associated with poorer outcomes (i.e., requiring therapeutic blood product intervention) (al-Mashhadani *et al.*, 1994). Hence, further research is needed to assess the value of pro-and anticoagulant molecules as prognostic biomarkers of heatstroke provoked organ injury/recovery using highly sensitive assays.

Von Willebrand factor antigen (vWfAg) and soluble thrombomodulin are likely the most studied biomarkers of endothelial cell injury activation or injury in heatstroke (Shieh *et al.*, 1995; Bouchama *et al.*, 1996b; Bouchama *et al.*, 2005; Huisse *et al.*, 2008; Tong *et al.*, 2014; Zhang *et al.*, 2018; Proctor *et al.*, 2020). vWfAg is synthesized and stored in endothelial cells and megakaryocytes, and mediates platelet aggregation and adhesion at vascular injury sites and endothelial damage (Lip & Blann, 1997). In an experimental model of heatstroke, immunostaining analysis revealed higher expression of vWfAg in most organs of the body, suggesting that the vascular endothelium activation is systemic (Roberts *et al.*, 2008b). However, no association between organ injury and survival was demonstrated in humans with heatstroke ($r^2=0.37$, $p>0.05$) (Bouchama *et al.*, 1996b).

Thrombomodulin is a transmembrane proteoglycan located on the surface of the vascular endothelium that functions as an anticoagulant (Martin *et al.*, 2013). There is also a

soluble form of thrombomodulin released from membrane-bound thrombomodulin that can be released in the circulation through proteolytic cleavage and is considered a marker of endothelial cell damage (Martin *et al.*, 2013). Thrombomodulin activates coagulation and inhibits tissue plasminogen activator-induced fibrinolysis following binding to thrombin and thrombin-activatable fibrinolysis inhibitor, respectively (Esmon & Owen, 1981; Bajzar, 2000). Recombinant thrombomodulin given to heat-stressed rodents prevented heatstroke by inhibition of HMGB1, a damage-associated molecular pattern indicating that thrombomodulin also has an anti-inflammatory function in heatstroke (Hagiwara *et al.*, 2010). Several clinical and experimental studies demonstrated an increased plasma soluble thrombomodulin concentration in heatstroke associated with severity and survival in some studies, but this was not consistently observed (Shieh *et al.*, 1995; Bouchama *et al.*, 2005; Huisse *et al.*, 2008; Tong *et al.*, 2014; Zhang *et al.*, 2018). Hence, further studies are needed to clarify the prognostic utility of soluble thrombomodulin in heatstroke.

Leucocyte trafficking—A central step in the complex crosstalk between inflammation, coagulation, and endothelial cells in heatstroke is the adherence of leukocytes and platelets to the endothelium via cell adhesion molecules. *Ex-vivo* study of leucocytes obtained from CHS patients showed that this process is mediated by the up-regulation of β2 integrin and down-regulation of L-selectin (Huisse *et al.*, 2008). Changes in lymphocyte β2 integrin expression were also demonstrated in vivo in both CHS and EHS (Hammami *et al.*, 1998b; Lu *et al.*, 2004b). Notably, soluble forms of adhesion molecules are released in the circulation and correlate with the concentration of molecules expressed on the cells and various diseases activity. Thus, soluble adhesion molecules may constitute more practical biomarkers in clinical settings (Gearing & Newman, 1993). In CHS patients, elevated plasma concentrations of soluble intercellular adhesion molecule 1, E-selectin, and L-selectin adhesion molecules have been documented (Bouchama *et al.*, 1996b; Hammami *et al.*, 1998a). L-selectin correlated significantly with E-selectin ($r=0.68$, $p=0.0002$) and the level of consciousness ($r=-0.45$, $p=0.03$) in heatstroke patients (Hammami *et al.*, 1998a). Compared with other soluble adhesion molecules, E-selectin is probably the most specific among these biomarkers of endothelial cell dysfunction and hence merits further study (Gearing & Newman, 1993).

Endothelial barrier integrity—Damage to the endothelium resulting in loss of its barrier function and vascular leaks were demonstrated in human and non-human primate models of heatstroke (Sohal *et al.*, 1968; Roberts *et al.*, 2008b). The luminal surface of the endothelium is covered by the glycocalyx, a network of membrane-bound proteoglycans and glycoproteins that binds and incorporates soluble molecules derived from the plasma and endothelium. Heatstroke disrupts the endothelial glycocalyx as assessed by plasma levels of syndecan-1 and hyaluronan in laboratory animals (Kobayashi *et al.*, 2018; Umemura *et al.*, 2018; Zhang *et al.*, 2018). Syndecan-1 is the principal component of the endothelial glycocalyx and hyaluronan maintains vascular integrity through endothelial glycocalyx modulation (Lennon & Singleton, 2011). Other biomarkers of endothelial cell activation and loss of integrity include the detachment of endothelial cells and their release into the peripheral circulation. An ~2.5 fold increase in circulating endothelial cells in an experimental heatstroke rat model has been demonstrated, suggesting it can be useful

as a marker of endothelial injury (Tong *et al.*, 2014). Overall, biomarkers of vascular barrier function in heatstroke were primarily established experimentally and deserve further translational studies

Brain

Heatstroke encephalopathy is a universal primary manifestation of heatstroke (Leon & Bouchama, 2011). Heatstroke induced encephalopathy is characteristically an early event in the natural course of this condition, preceding the dysfunction of other organ-systems. Further, heatstroke encephalopathy is associated with long-term cognitive and motor disability in heatstroke survivors (Dematte *et al.*, 1998; Argaud *et al.*, 2007; Yang *et al.*, 2017). These findings suggest that the brain is extraordinary vulnerable to heatstroke. The neurologic signs and symptoms are not specific and include, altered mental status, delirium, and coma (Leon & Bouchama, 2011; Casa *et al.*, 2012; Bouchama *et al.*, 2022a). There are no lateralizing signs on physical examination and brain imaging at presentation is normal with no evidence of structural damage (Leon & Bouchama, 2011). In contrast, follow-up brain imaging performed months after heatstroke in patients with persistent central nervous system dysfunction can reveal damage to the cerebellum, prefrontal cortex, or hippocampus (Leon & Bouchama, 2011). Hence, this indicates that early brain injury leading to delayed brain damage may not be readily detectable with standard diagnostic approaches. In this context, biomarkers would aid in the early detection of injury and predict delayed brain damage would be crucial. Notably, S100 calcium-binding protein β (S100β) (Chun *et al.*, 2019; Li *et al.*, 2020) and neuron-specific enolase (NSE) (Li *et al.*, 2020) have been recently proposed as potential biomarkers of heatstroke encephalopathy.

S100β, a glial-specific protein expressed by astrocytes and Schwann cells, functions as a neurotrophic and neuronal survival factor in the developing brain. S100β can be released in the circulation from injured cells (Van Eldik *et al.*, 1986). Accordingly, S100β levels in serum and cerebrospinal fluid have been used as biomarkers of brain and blood brain barrier injury in many conditions, including traumatic brain injury, subarachnoid hemorrhage, and stroke (Persson *et al.*, 1987). Exercise in the heat increases circulating S100β indicating that heat stress may alter blood-brain permeability (Watson *et al.*, 2005). However, additional work refutes these findings during exercise in the heat (Cheuvront *et al.*, 2008) and passive heat stress (Shepley *et al.*, 2021) models in humans. Nonetheless, two recent studies in heatstroke patients demonstrated elevated S100β protein in the serum and cerebrospinal fluid of heatstroke patients (Chun *et al.*, 2019; Li *et al.*, 2020), with one study finding that the heatstroke patients with poor prognosis (defined as not being able to live without assistance at hospital discharge) having serum S100β concentrations ~5 times higher than in heatstroke patients with a good prognosis (Chun *et al.*, 2019). NSE is a dimeric isoform of the glycolytic enzyme enolase expressed mainly in neurons (Kaiser *et al.*, 1989). NSE is upregulated in injured neuronal cells and can spill over into the circulation from injured or dying cells (Persson *et al.*, 1987). Elevated NSE levels in the serum and cerebrospinal fluid have been reported in various brain pathologies, such as head trauma and vascular stroke (Persson *et al.*, 1987). In heatstroke patients, NSE levels strongly correlated with neurological outcomes for up to seven days post-heatstroke (all days $r \geq -0.624$, all days $p < 0.05$), suggesting that NSE could be helpful as diagnostic and prognostic biomarker of

heatstroke encephalopathy (Li *et al.*, 2020). Overall, these observations indicate that S100β and NSE are promising biomarkers for early detection or follow-up of heatstroke associated brain injury and thus merit larger prospective studies to determine their sensitivity and specificity.

Kidneys

Kidney damage is well recognized as one of the hallmarks of multiorgan injury caused by heatstroke (Leon *et al.*, 2006b; Roberts *et al.*, 2008a; King *et al.*, 2015). Indeed, patients diagnosed with heatstroke often suffer from acute kidney injury (e.g., (Gauss & Meyer, 1917; Kew *et al.*, 1967; Schrier *et al.*, 1970)), characterized by elevations in serum creatinine, which is indicative of reductions in glomerular filtration rate, measured as part of the basic clinical bloodwork (Ward *et al.*, 2020). A recent analysis of 187 EHS cases in southern China identified that ~44% of patients developed acute kidney injury and that ~27% of the patients with acute kidney injury died within 90 days (Wu *et al.*, 2021). Thus, the management of acute kidney injury following the diagnosis of heatstroke is an essential consideration. It is important to note, however, that acute kidney injury is not ubiquitous such that other observations indicate that in most heatstroke cases, elevations in serum creatinine (Ward *et al.*, 2020) and other markers of renal dysfunction (Kew *et al.*, 1967; Schrier *et al.*, 1970) subsided as soon as 16 days following heatstroke.

Data in animal models of heatstroke indicate that kidney injury is contributed to by vascular congestion, hemorrhage, and thrombi distributed throughout renal tissues (Leon *et al.*, 2006b; Roberts *et al.*, 2008a; King *et al.*, 2015). This likely occurs secondary to the heatstroke-induced systemic inflammation and coagulation activation that catalyze hemorrhagic and thrombotic events within the kidneys (and other organs), similar to sepsis (Ma *et al.*, 2019). Recent findings support that the etiology of kidney injury during heatstroke also includes injury to the renal tubules (Lin & Zhang, 2019). This study additionally identified serum neutrophil gelatinase-associated lipocalin (NGAL) as a potential early biomarker of kidney injury secondary to EHS, such that elevations in serum NGAL were almost 10 times higher post EHS compared to a non-heated control group (Lin & Zhang, 2019). Such observations are consistent with findings in humans undertaking exercise in the heat (Schlader *et al.*, 2017). While NGAL, which functions as a bacteriostatic agent secondary to tissue injury, is primarily expressed in renal tissues, it is also expressed in liver and heart tissues, suggesting that elevations in circulating NGAL may not be specific to the kidneys (Chapman *et al.*, 2020a). This issue may be overcome by examining urinary NGAL or other markers of urinary tubular injury, such as kidney injury molecule-1, insulin-like growth factor binding protein-7, and/or tissue inhibitor metalloproteinase-2 (Chapman *et al.*, 2020a). Indeed, urinary NGAL is elevated in dogs suffering from heatstroke (Segev *et al.*, 2015). Whether this observation translates to humans and whether urinary markers of kidney injury can be used as biomarkers of kidney injury during or following heatstroke is unknown and warrants further study. It is also worth highlighting that obtaining urine samples during/following heatstroke may be challenged because urine production is reduced by elevations in body temperature and hypovolemia (Chapman *et al.*, 2020a). Thus, urinary kidney injury biomarkers may only be available upon urinary catheterization. Moreover, the specificity of plasma or urinary NGAL towards heatstroke should be examined, as they

are both temporarily elevated during heat stress in humans without heatstroke and these increases resolve within 24 hours (Schlader *et al.*, 2017; Chapman *et al.*, 2020b).

Gastrointestinal system

Intestines

The systemic immune response to heatstroke has been partially attributed to the development of intestinal cellular dysfunction, making the gastrointestinal tract leaky, and allowing for the translocation of endotoxin into the systemic circulation (Hall *et al.*, 1999; Novosad *et al.*, 2013). The mechanisms by which the gastrointestinal tract may contribute to heatstroke etiology are outside of the scope of this review. Here, however, we consider the consequences of heatstroke on biomarkers of intestinal injury and recovery. To this end, it is notable that diarrhea is consistently observed in heatstroke (Hart *et al.*, 1980), which typically subsides with cooling (Leon & Bouchama, 2011; Bouchama *et al.*, 2022a). Data from preclinical models of CHS show severe intestinal injury in the duodenum, jejunum, and ileum (Novosad *et al.*, 2013) that is characterized by cellular apoptosis (Roberts *et al.*, 2008a) and interstitial edema (Miyamoto *et al.*, 2021). Whether these findings are consistent in the clinical setting remains unknown (Bouchama *et al.*, 2022a). However, circulating intestinal fatty acid-binding protein 2 (I-FABP), a protein found in enterocytes of the small intestinal epithelium that when found in the circulation is often interpreted as evidence of increased intestinal permeability and/or injury (Wells *et al.*, 2017). I-FABP is elevated in patients with heatstroke, with peak responses occurring three days post-heatstroke and reduced with treatment (Zhang *et al.*, 2015), suggesting that I-FABP may be a biomarker of gastrointestinal injury associated with heatstroke. The experimental demonstration of an increased I-FABP gene expression in intestinal mucosa up to 7 h post-passive heat exposure (Miyamoto *et al.*, 2021) (Miyamoto et al., 2021) and elevations in plasma I-FABP protein levels for 24 h after EHS (King *et al.*, 2015), all of which were associated with intestinal histopathological damage, lend support to this interpretation. Given these I-FABP observations, other biomarkers of increased intestinal permeability (e.g., urinary lactulose/rhamnose ratio) may also be considered as intestinal injury biomarkers following heatstroke. However, data from recent meta-analyses indicate that hyperthermia (Pires *et al.*, 2017) and exercise (Chantler *et al.*, 2021), in the absence of heatstroke, consistently increases markers of intestinal permeability, including I-FABP, casting doubt on the value of I-FABP and other markers of intestinal permeability on the specificity of heatstroke-induced intestinal injury or recovery.

Liver

Liver dysfunction and injury are relatively common following heatstroke (Leon & Bouchama, 2011). Indeed, increases in circulating alanine aminotransferase (ALT) and aspartate aminotransferase (AST), standard clinical tests of liver function, are ubiquitous in heatstroke patients (Ward *et al.*, 2020). Moreover, data from a mouse model of EHS (King *et al.*, 2015) and a baboon model of CHS (Roberts *et al.*, 2008a) indicate that heatstroke induces vascular congestion and hemorrhagic thrombosis in liver tissues, while postmortem clinical findings provide support for liver tissue ischemia and necrosis (Malamud *et al.*, 1946b). Notably, these pathological findings also identified that the extent of liver damage

was greater when survival following heatstroke exceeded 30 hours (Malamud *et al.*, 1946b), suggesting that the etiology of liver damage initiated by heat cytotoxicity is likely amplified (or worsened) by ischemia-reperfusion injury and an exacerbated systemic inflammatory response. This interpretation is supported by the timing of changes in ALT and AST following heatstroke, with peak concentrations being observed two to three days post-heatstroke (Ward *et al.*, 2020; Ji *et al.*, 2021). There are no formally identified biomarkers, as defined herein, quantifying the extent of liver damage or recovery post-heatstroke. Nevertheless, it is important to recognize that recent evidence supports that increased circulating clinical markers of hepatic dysfunction, including ALT and AST, are associated with an increased likelihood of EHS mortality (Ji *et al.*, 2021; Li *et al.*, 2022). However, it is unknown whether this is due to hepatic causes per se or simply reflects the more severe clinical condition of these patients (Ji *et al.*, 2021). This conclusion is supported by evidence that heatstroke elicits liver injury via interleukin-1β mechanisms (Geng *et al.*, 2015) and that pretreatment with specific antibodies that inhibit circulating HMGB1 protein, which is at least partially implicated in the inflammatory response during EHS (Tong *et al.*, 2011a), alleviates CHS-provoked liver pathology (Tong *et al.*, 2013).

Lungs

Acute lung injury (ALI) is common and is associated with excessive systemic inflammation and coagulation activation in heatstroke (El-Kassimi *et al.*, 1986). However, whether ALI is a direct consequence of excessive heat, or a secondary consequence of systemic inflammation and coagulation activation remains unclear. Several studies in rats used ambient heating to induce CHS and the associated multiorgan failure syndrome that is commonly observed in clinical patients (Yang *et al.*, 2009; Hsi-Hsing *et al.*, 2010). The specific experimental manipulation included exposure of the rats to a thermally stressful environment of 42°C and 80% relative humidity, in which the ambient air had a heat content of approximately 153 kJ/kg. With 146 kJ/kg of heat content at the respiratory surface (assuming 100% RH and a normal body temperature of 37.9°C), this experimental design not only effectively prevents any heat dissipation from the experimental subjects but may also result in *net heat absorption* from the environment into the respiratory tissues. This pattern of preferentially heating the respiratory tract, with the rest of the body accumulating heat due to perfusion of heated blood from the respiratory tract, is likely consistent with the instances of CHS but differs from EHS. Particularly relevant in animal models of heatstroke is that the mechanism of selective brain cooling relies on cooling blood in the upper respiratory tract. Thus, it can be speculated that this arrangement may lead to faster central nervous system heating and signs of heatstroke than in typical clinical situations in which the primary source of heat is other tissues in the body. In contrast, studies of CHS using a lower ambient temperature failed to show any histological evidence of lung injury (Leon *et al.*, 2006a), supporting the contention that early ALI in the development of heatstroke may be a function of selective injury to the lung during conditions favoring heat uptake through that organ. In contrast, ALI developing later in the disease process may be secondary to systemic inflammatory processes and not specific to heat injury. As a result, biomarkers specific to ALI are probably relevant only in this context.

Skeletal muscle

As the principal source of excess heat, skeletal muscle plays a central role in EHS. Biomarkers of skeletal muscle injury, such as myoglobin and creatine kinase, are prominent in cases of exertional heatstroke, with reported increases of 2 to 4.5 times, respectively, above normal limits in a retrospective study of cases of EHS (Laitano *et al.*, 2021). However, cases of exertional rhabdomyolysis without associated heatstroke can demonstrate similar or higher serum concentrations of these biomarkers (Lippi, 2019), making these biomarkers specific to muscle injury but not specific for the underlying cause of muscle injury. Similarly, serum cytokine concentrations lack specificity regarding skeletal muscle injury in heatstroke since it has been well-established that, skeletal muscle has considerable secretory activity during exercise (Pedersen & Febbraio, 2008). Given that skeletal muscle activity is inherently associated with increased skeletal muscle temperature due to the metabolic activity needed to support skeletal muscle contraction, it is impossible to separate the effects of exercise from the thermal consequences in this tissue. Furthermore, the same cytokines released by active skeletal muscles can also be released by other tissues, making it less certain that the cascade of cytokines commonly identified in cases of heatstroke is of muscular origin. Thus, these biomarkers may be more specific to exertion (and perhaps the heating of the skeletal muscle tissue per se) than whole-body hyperthermia secondary to an inability to dissipate the heat produced by the skeletal muscle. That said, an understanding of the extent of skeletal muscle injury may have important implications regarding the management of kidney injury during or following heatstroke.

Skeletal muscle also can suffer injury and presumably release biomarkers of that injury during CHS. A specific role for mitochondria in heat-induced muscle injury is suggested by the fact that lesions induced by passive heating are more prominent in muscles with higher oxidative capacity (Sharma *et al.*, 2021). Increased temperature has been shown to increase mitochondrial leak respiration in skeletal muscle in rats (Jarmuszkiewicz *et al.*, 2015), dogs (Davis & Barrett, 2021), horses (Davis *et al.*, 2020), and humans (Fiorenza *et al.*, 2019). Increased leak respiration will decrease the efficiency of ATP synthesis and as a result increase heat production through oxidative metabolism is necessary to maintain the desired rate of ATP synthesis. If heat dissipation from the tissue is not increased, a cycle will develop resulting in skeletal muscle overheating and metabolic failure. Additional possible consequences of skeletal muscle heating that may result in damage are the production of reactive oxygen species, which is slightly increased during phosphorylating respiration in hyperthermic rat muscle (Jarmuszkiewicz *et al.*, 2015). Finally, tissue hyperthermia may cause failure of cytoplasmic calcium regulation due to heat-induced dysfunction of the sarcoplasmic calcium-mediated calcium channel (aka, ryanodine receptor RyR1) (Capacchione & Muldoon, 2009), contributing to a syndrome of malignant hyperthermia. Due to these mechanisms, the presence of biomarkers of skeletal muscle injury in the absence of a history of recent exertion could support the passive accumulation of heat – and by extension, systemic heat stress or heatstroke – as an important differential diagnosis.

Summary

Based on observational evidence indicating that a single episode of heatstroke could have harmful acute and chronic long-term health outcomes across numerous organ systems, this narrative review aimed to identify biomarkers of heatstroke-induced organ injury and repair that could be used to guide further development and utilization. This includes as a potential aid to initiate specific organ-protective strategies and prognostication (Figure 1). To this end, we have identified numerous biomarkers related to many aspects of generalized heatstroke induced cellular injury and tissue damage, and heatstroke provoked cardiovascular, renal, cerebral, intestinal, and skeletal muscle injury (Figure 2). The literature supporting biomarkers related to vascular endothelium dysfunction following heatstroke appears to be the most extensive, which may be related to the uniquely multi-functional nature of the endothelium. Moreover, no novel biomarkers were identified for liver or lung injury, which may be because it remains unclear whether injury to these organs is specific to heatstroke or generalized systemic inflammation. In general, there was evidence that the identified biomarkers were specific to acute organ injury. However, the kinetic profile of these biomarkers post-heatstroke, and their reproducibility and specificity to predict organ recovery or failure and long-term outcomes remain relatively unexplored. Collectively, before the translation of the identified biomarkers into clinical practice, additional preclinical and clinical prospective studies are required to better understand the clinical utility of these biomarkers. These studies should likely focus on the relations between the identified biomarkers and how they relate to long-term post-heatstroke health outcomes.

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What is the topic of this review?

This narrative review provides an up-to-date overview of the status and potential role of novel biological markers (biomarkers) that can help identify the patients at risk of organ injury or long-term complications following heatstroke.

What advances does it highlight?

This narrative review has identified numerous biomarkers related to many aspects of generalized heatstroke induced cellular injury and tissue damage, and heatstroke provoked cardiovascular, renal, cerebral, intestinal, and skeletal muscle injury. No novel biomarkers were identified for liver or lung injury.

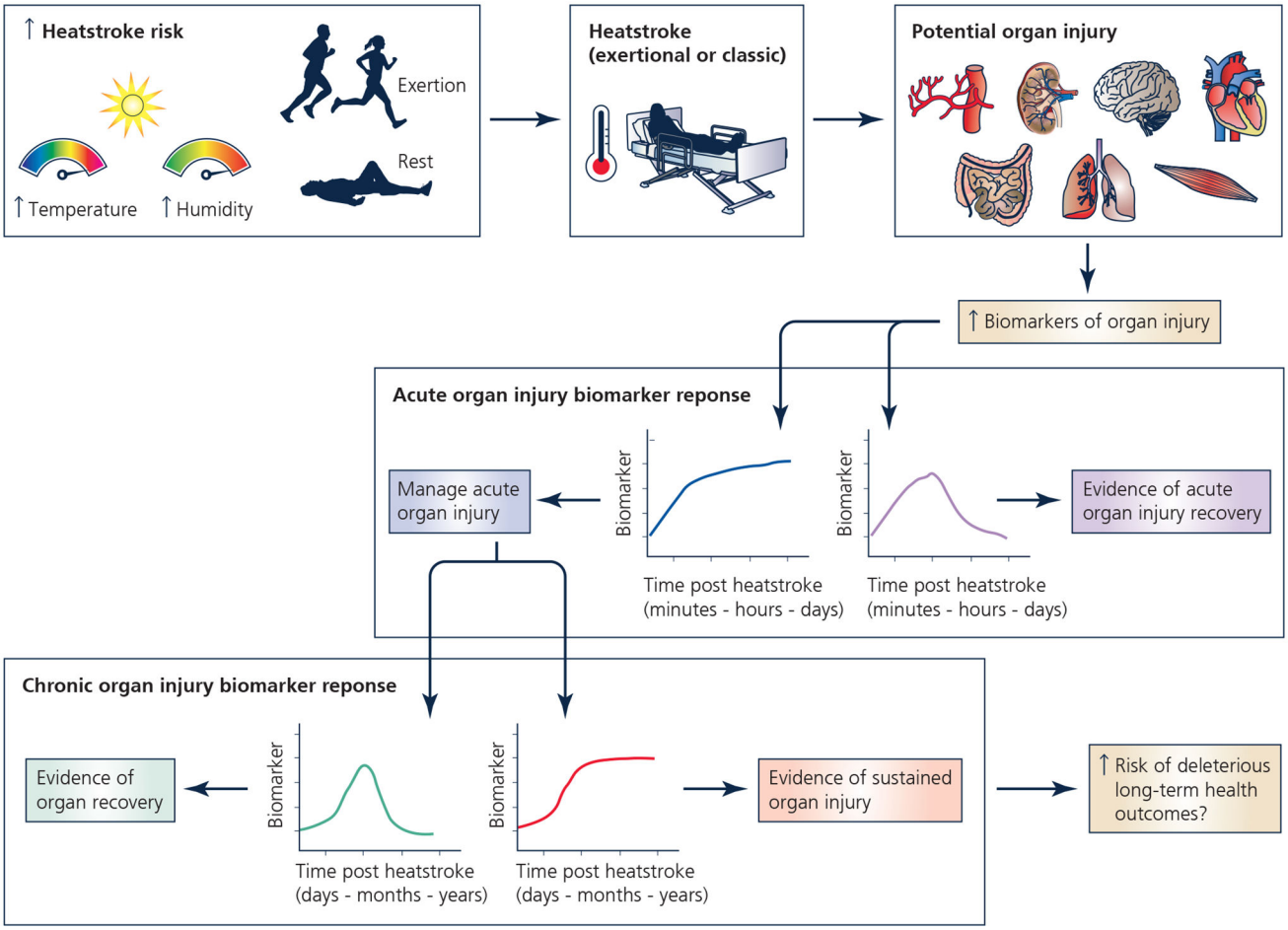

Figure 1:

Potential utility of biomarkers to detect tissue and organ injury, monitor recovery, and predict long-term complications following heatstroke. The purpose of this narrative review is to identify biomarkers of heatstroke induced organ injury and repair. Created with BioRender.com.

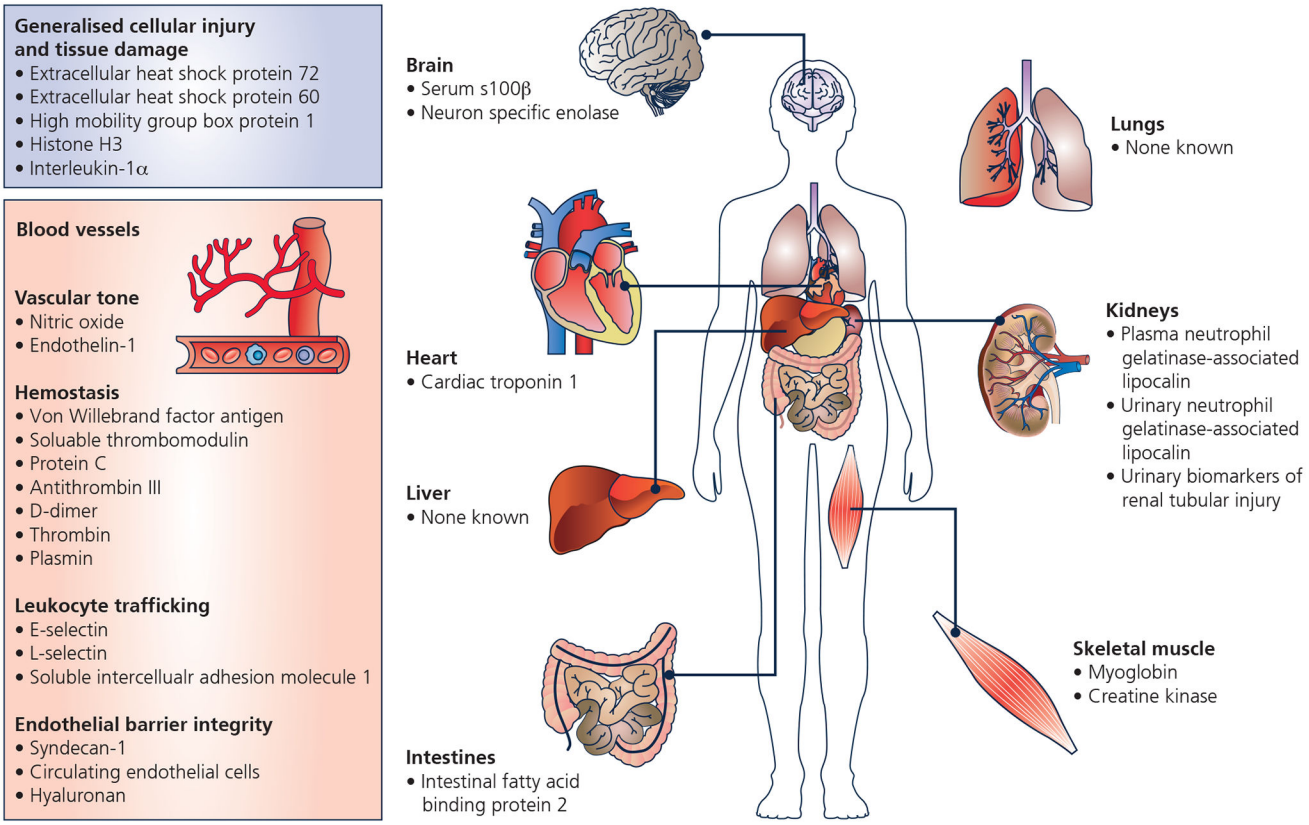

Figure 2:
Identified biomarkers of heatstroke induced organ injury and/or recovery. Created with BioRender.com.