Journal of Thermal Biology 85 (2019) 102423

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: http://www.elsevier.com/locate/jtherbio

Gene expression profiling of humans under exertional heat stress: Comparisons between persons with and without exertional heat stroke

Ming Qiang Ren [a,b], Josh B. Kazman [a,b,*], Preetha A. Abraham [a,b], Danit Atias-Varon [c], Yuval Heled [c], Patricia A. Deuster [a]

[a] Consortium for Health and Military Performance, Department of Military & Emergency Medicine, F. Edward Hébert School of Medicine, Uniformed Services University, USA

[b] Henry M. Jackson Foundation for the Advancement of Military Medicine, USA

[c] Heller Institute of Medical Research, Sheba Medical Center, Tel Hashomer, Israel

A B S T R A C T

Exertional heat stroke (EHS) is a leading cause of preventable morbidity and mortality among both athletes and warfighters. Therefore, it is important to find blood biomarkers to predict susceptibility to EHS. We compared gene expression profiling from blood cells between two groups of participants – those with and those without a history EHS – by using genome-wide microarray analysis. Subjects with a history of EHS (n = 6) and non-EHS controls without a history of EHS (n = 18) underwent a heat tolerance test and a thermoneutral exercise challenge on separate days. The heat tolerance test comprised of 2-h of walking, at 5 km/h and 2% incline, with ambient conditions set at 40 °C, 40% relative humidity; the thermoneutral test was similar, but had ambient conditions set at 22 °C. Next, we examined gene expression profiles, quantified based on arithmetic differences (post minus pre) during the heat test minus changes during the thermoneutral test. Genes related to interleukins and cellular stress were significantly down-regulated in participants with a history of EHS compared to their non-EHS counterparts. Suppression of these genes may be associated with susceptibility to exertional heat injury. Prospective research is required to determine whether similar gene expression profiling can be potentially used as blood biomarkers to predict susceptibility to EHS.

1. Introduction

Exertional heat stroke (EHS) poses a regular threat to various populations such as athletes and military service members/warfighters. EHS typically occurs during strenuous activity in the heat, when the environmental heat load and internal heat production overwhelm the body's ability to thermoregulate. Key intrinsic risk factors for EHS include high percent body fat and low cardiorespiratory fitness (Bedno et al., 2014). Still, EHS remains difficult to predict because it often occurs in the most highly fit and motivated individuals who are performing very competitive activities (Shibolet et al., 1976). In fact, a history of previous EHS is one of the strongest predictors of EHS in the military (Nelson et al., 2018); an observation that has increased scrutiny with regard to returning warfighters back to duty following EHS (O'Connor et al., 2010).

Deciding whether to return warfighters back to duty following EHS is often a subjective process, although algorithms to assist with this clinical decision have been proposed (Kazman et al., 2013). Heat tolerance testing (HTT) can help with the decision-making process as it assesses whether a patient demonstrates an appropriate or compensable response to heat stress. The most widely used HTT, developed by the Israeli Defense Forces, is a standardized 2-h walking test on a treadmill (at 5 km/h and 2.0% incline) in an environmental chamber with ambient conditions set at 40 °C and relative humidity at 40% (Moran et al., 2007). Published evidence suggests that the Israeli HTT is a useful tool for making return to duty decisions (Epstein et al., 2012; Kazman et al., 2013), although some experts remain skeptical about the applicability of heat tolerance to EHS risk in general (O'Connor et al., 2010) and/or the specific parameters of the Israeli HTT in particular (Stearns et al., 2018). Ideally, one would be able to collect a biologic sample prospectively, which could indicate susceptibility to EHS. To date, a few studies have attempted to address EHS susceptibility through molecular approaches, in particular genetics (Heled et al., 2004; Li et al., 2014), but no definitive markers have yet been established. Thus, research into biomarker pathways is important. What is well established through cell culture and animal studies is that marked heat exposure is associated with cellular stress as indicated by upregulation of various cytokines, excessive production of reactive oxygen species, disruption in the coagulation system, and systemic inflammatory responses (Bouchama and Knochel, 2002; King et al., 2017; Leon and Helwig, 2010; Yu et al., 2018, 2019). Some

* Corresponding author. Henry M. Jackson Foundation for the Advancement of Military Medicine, 6720B Rockledge Drive, Office 669, Bethesda, MD, 20817, USA.
E-mail address: josh.kazman.ctr@usuhs.edu (J.B. Kazman).

https://doi.org/10.1016/j.jtherbio.2019.102423
Received 14 March 2019; Received in revised form 20 August 2019; Accepted 24 September 2019
Available online 25 September 2019

evidence suggests the cytokine response actually confers a protective role for resolution of inflammation (King et al., 2017; Leon et al., 2006; Phillips et al., 2015). We believe that studies investigating expression of various pathways might elucidate physiologic mechanisms underlying heat tolerance. Such research might also be used to predict susceptibility to EHS and perhaps to the HTT (Stearns et al., 2018).

In this paper, we combined a human model of exertional heat-stress with global gene expression profiling to characterize transcriptomic responses of peripheral blood mononuclear cells (PBMCs) before and after a HTT.

2. Methods

2.1. Participants

Two groups of participants were recruited: those without a history of EHS (non-EHS controls) from university and military communities around the DC-metropolitan area in the United States and those who had EHS were referred by physicians. This study was approved by the Institutional Review Board at the Uniformed Services University of the Health Sciences, and all participants provided written informed consent in agreement with the Helsinki Declaration. All participants: 1) were 18–45 years of age; 2) had a waist circumference <39.4 inches (100 cm); 3) were not hypertensive (resting systolic and diastolic blood pressure < 140 and < 90 mmHg, respectively); 4) had no previous history of malignant hyperthermia; 5) were not pregnant or lactating; 6) were not anemic; 7) were not using glucose-lowering agents, prednisone, or b-blockers; 8) had no history heart disease; and 9) were not presently being treated for any mental health disorder.

Out of 69 participants (20 with a history of EHS) who completed all exercise tests, we had complete and quality biomarker data on 41 participants. Next, among the 20 EHS participants, we selected those who had self-reported multiple incidents of exertional heat illness (n = 5) or had a typical presentation of EHS (n = 1). Eighteen participants without a history of EHS were then selected to create roughly equivalent groups along baseline characteristics (percent body fat/%BF, maximal aerobic capacity/VO2max) and HTT outcomes (see below). As such, we evaluated biomarkers for 24 participants.

Those with a history of EHS could be enrolled in the study no sooner than six weeks after a previous medically-confirmed incident of EHS. Out of the six patients selected in this sample, their previous EHS was on average 204 ± 107 days prior to testing, and the range was 87–336 days. In addition to the prior medically-confirmed incident EHS event, five also reported other incidents of exertional heat illness: three reported a second prior incident; one reported two prior incidents; and one reported three prior incidents. All participants provided informed consent before participating in the study, underwent a baseline evaluation, and then completed exercise tolerance tests.

2.2. Baseline evaluation

Participants underwent a medical examination, anthropometric evaluations (body mass, height, waist circumference, and %BF), and a maximal aerobic graded exercise test to assess maximal aerobic power (VO_{2max}). Body mass was measured with a calibrated metric scale to the nearest 0.1 kg, and height was measured to the nearest 0.1 cm. %BF was assessed by using the InBody720 (InBody CO., Cerritos, CA), a multi-frequency bioelectrical impedance analyzer (Ogawa et al., 2011). Maximal aerobic power was determined by using open-circuit spirometry for monitoring expired respiratory gases (Oxycon Mobile portable system, Viasys Healthcare Inc, Yorba Linda, CA). The test protocol, previously described by our laboratory (Kyle et al., 1989), consisted of a 5-min warm-up (5.0 km/h and a 2.0% grade) and then running at a constant speed between 7.7 and 13.7 km/h (based on HR achieved during warm-up). The incline started at 0% and increased 2.5% every 2 min until the participant could no longer continue or VO_2 plateaued with an increase in workload.

2.3. Exercise tolerance tests

HTT is performed according to the following protocol: 120 min exposure to 40 °C and 40% relative humidity in a climatic chamber while walking on a treadmill dressed in shorts and T-shirt at a pace of 5 km/h and 2% grade. Rectal temperature (Tr) and heart rate (HR) are continuously monitored, and sweat rate is calculated. Participants are classified as "heat intolerant" if, during the test, their Tr exceeds 38.5 °C, their HR exceeds 150 bpm, or their Tr continues to increase throughout the second half of the test (based on a rise of Tr > 0.45 °C during the second hour) (Amit et al., 2013; Moran et al., 2007).

Two tolerance tests were conducted on separate days, with the order randomized: a HTT (ambient temperature: 40 °C; 40%RH) and a thermoneutal tolerance test (TTT; ambient temperature: 22 °C; 40%RH). Aside from ambient temperature, TTTs followed the format of the HTT, as previously described: participants walked on a treadmill at 5.0 km/h at a 2% grade for 120 min; wore light clothing; and were all tested in the morning (Moran et al., 2007). Additionally, women were tested between days three and nine of their follicular phase to control for menstrual phase differences in basal body temperature. For HTTs and TTTs, urine specific gravity (USG) was measured and had to be less than 1.02 prior to starting to ensure adequate hydration. Participants with elevated USG (n = 8 for HTT, and n = 5 for TTT) were asked to drink water; testing was delayed, and USG was re-evaluated. Average values for pre- and post-exercise USG are provided in Table 1. During testing, participants were encouraged to hydrate with water *ad libitum* (up to one L/hr). Tr was measured by using a rectal thermometer inserted 10 cm beyond the anal sphincter connected to a thermocouple data acquisition system (Type T Thermocouples with Thermes WiFi, Physitemp, Clifton, NJ). Key metrics from the test included maximal HR (Polar Team 2 Pro, Polar USA Inc, Lake Success, NY) and maximal Tr. Maximal physiological strain index (PSI) was also calculated from changes in HR and Tr over testing, as suggested by Moran et al. (1998). We did not observe any influence of season of testing on HTT outcomes. Throughout the study, HTTs were conducted across all seasons, including the spring (controls: 40%; patients: 17%), summer (controls: 28%; patients: 17%), fall (controls: 22%; patients: 33%), and winter (controls: 11%; patients 33%). Across all participants, season had no significant effect on maximal Tr (F [df: 3, 20] = 1.6, $p = 0.21$), HR (F [df: 3, 20] = 0.3, $p = 0.82$), and PSI (F [df: 3, 20] = 0.2, $p = 0.89$), and did not significantly contribute to models predicting these outcomes after controlling for VO_{2max} and %BF.

2.4. Blood sample collection and procession

Venous whole blood was collected before and immediately after the experimental test by using an indwelling venous catheter and a 24-inch extension that protruded from the sleeve of the protective ensemble. Blood samples were drawn into sterile syringes and immediately transferred into corresponding vacutainer collection tubes coated with EDTA (BD Biosciences). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation of PBS-diluted (1:1) blood samples over a Ficoll-Hypaque gradient (Sigma-Aldrich) following the manufacturer's instructions. The total RNA from PBMC was isolated by using QIAamp RNA Blood Mini Kit according to the manufactural manual.

2.5. Whole genome RNA microarray

Whole-genome expression (RNA) was analyzed in all pre- and post HTT samples by using Illumina cDNA-mediated annealing, selection, extension and ligation (DASL) processes as described by the manufacturer (Illumina Inc., San Diego, CA). Messenger RNA microarrays were performed using Illumina HumanHT-12 v4 BeadChip technology. Each chip contains 31,000 annotated genes and 47,000 probes derived from

Table 1
Participant characteristics, overall and based on history of exertional heat stroke (mean ± SD, or %).

Variable	Total (n = 24)	History of EHS (n = 6)	No EHS (n = 18)
Age, y	26 ± 5	25 ± 4	27 ± 6
Women, n (%)	7 (29%)	0 (0%)	7 (39%)
Race/Ethnicity, n (%)			
Caucasian	21 (88%)	6 (100%)	15 (83%)
Asian	2 (8%)	0 (0%)	2 (11%)
Hispanic	1 (4%)	0 (0%)	1 (6%)
Heat Intolerant, n (%)[a]	5 (21%)	0 (0%)	5 (28%)
Anthropometric and Physiologic Measures			
Height (cm)	175 ± 10	182 ± 4	172 ± 10*
Weight (kg)	79.2 ± 15.3	93.6 ± 6.3	74.4 ± 14.4*
Body Mass Index (kg/m^2)	25.7 ± 3.1	28.5 ± 1.4	24.8 ± 2.9*
Body Surface Area (m^2)	1.94 ± 0.23	2.14 ± 0.09	1.87 ± 0.22
Body Surface-to-Mass Ratio (m^2·kg^{-1}·10^2)	2.50 ± 0.21	2.29 ± 0.07	2.55 ± 0.20*
Percent Body Fat (%)	17.7 ± 6.4	15.3 ± 3.9	18.5 ± 7.0
Waist Circumference (cm)	81.2 ± 8.9	89.3 ± 2.9	78.4 ± 8.5*
VO_{2max} (mL·kg^{-1}·min^{-1})	48.4 ± 8.8	46.5 ± 7.7	49.1 ± 9.2
Heat Test (maximal values)			
Tr (°C)	38.0 ± 0.29	38.0 ± 0.29	37.9 ± 0.29
HR (bpm)	124 ± 21.6	121 ± 18.1	125 ± 23.0
PSI	4.6 ± 1.0	4.6 ± 1.0	4.6 ± 1.0
Sweat Rate (L·hr^{-1})	1.03 ± 0.40	1.03 ± 0.26	1.03 ± 0.44
Pre-USG	1.014 ± 0.006	1.018 ± 0.015	1.013 ± 0.007
Post-USG[c]	1.014 ± 0.007	1.012 ± 0.005	1.015 ± 0.008
Thermal-Neutral Test (maximal values)[b]			
Tr (°C)	37.5 ± 0.34	37.6 ± 0.40	37.5 ± 0.33
HR (bpm)	104 ± 14.8	98 ± 12.8	105 ± 15.3
PSI	2.6 ± 0.9	2.4 ± 1.0	2.7 ± 0.9
Sweat Rate (L·hr^{-1})	0.50 ± 0.33	0.54 ± 0.40	0.48 ± 0.31
Pre-USG	1.013 ± 0.005	1.011 ± 0.004	1.014 ± 0.005
Post-USG[c]	1.014 ± 0.007	1.015 ± 0.004	1.012 ± 0.008

EHS, exertional heat stroke; PSI, physiological strain index.
[a]Heat intolerance criteria included Tr ≥ 38.5, HR ≥ 150, or ΔTr in second hour of HTT ≥ 0.45 °C.
[c]Measured 60 min after exercise; during this period, *ad libitum* water consumption occurred.
*Group differences are statistically significant at $p < 0.05$.

the National Center for Biotechnology Information Reference Sequence RefSeq Release 38. The samples were applied randomly on the BeadChips (1 assay per sample). Arrays were scanned by using the HiScanSQ system and decoded images analyzed by GenomeStudio gene expression module (Illumina Inc.). Probes with signals that fulfilled the criteria of the Illumina probe detection *p*-value of 0.05 were considered different.

2.6. Gene expression data analysis

To minimize bias from differences of sample size, gender, and heat tolerant/intolerant, matched analysis was used to compare HTT versus TTT within identical participants, i.e. subtraction of gene expression level (log2 transformation) of pre- and post-tolerance test for each individual participant. The following formula was used: ΔHTT = HTTpost – HTTpre; ΔTTT = TTTpost – TTTpre; $\Delta\Delta EHS = \Delta HTT_{EHS}\text{-}\Delta TTT_{EHS}$; $\Delta\Delta Control = \Delta HTT_{Control}\text{-}\Delta TTT_{Control;}$ where EHS = participants with history of heat stroke and Control = participants with no history of EHS.

Differential expression analysis was performed using GenePattern software (software.broadinstitute.org/cancer/software/genepattern) with the "ComparativeMarkerSelection" module; a number of permutations of 0 were used to calculate asymptotic *p* values, as recommended when there are less than 10 samples in any class, and two sided T-tests were used to calculate *p* values. We used T-distributed Stochastic Neighbor Embedding (t-SNE) to construct probability distributions and visualizations of our pairwise data (van der Maaten and Hinton, 2008). The following parameters were used for t-SNE plots: perplexity 5, max iterations at default of 1000, and initial dimensions at 5 and theta 0.1.

3. Results

3.1. Physiological characteristic of sample and responses to HTT

The physiological characteristics of all of the participants are presented in Table 1. EHS patients had significantly greater BMI and waist circumferences than non-EHS controls, as shown in Table 1. They also had significantly lower body surface-to-mass ratio. Compared to non-EHS controls, EHS patients on average had slightly lower %BF and slightly higher VO_{2max}, but these differences were not statistically significant. Within the six EHS participants, duration since previous heat illness appeared to relate to their fitness level: Three participants had EHS within the previous four months, and they had higher VO_{2max} (51.5 ± 7.7 mL/kg/min) than the three participants who had EHS ten or more months ago (VO_{2max}: 41.6 ± 4.2 mL/kg/min), although %BF and age were similar across these two groups. Some non-EHS controls (28%) were heat intolerant as defined by the HTT criteria, relative to 0% of heat patients. However, on average, the two groups had comparable responses to the HTT and TTT: maximal Tr and HR during the HTT and TTT did not differ. In contrast, within-subject changes between the HTT and TTT differed significantly for Tr (*Cohen's* $d = 1.81$), HR ($d = 1.28$), PSI ($d = 1.78$) and sweat rate ($d = 1.15$).

3.2. Gene expression profiling differs between EHS and control groups

To investigate potential differences in gene expression profiles between EHS participants and non-EHS controls, we performed genome-wide microarray analysis of their PBMC samples (see Materials and Methods). Visual results of t-SNE analysis of profile datasets clearly showed separation of HTT and TTT tests (Fig. 1A). Each point on the t-SNE map represents an individual participant and each participant is colored based on its sub-cluster. This pattern of results suggests that exercise in a hot environment can clearly affect PBMC gene expression. To investigate gene expression profiling differences between participants with a history EHS and non-EHS controls, we further performed hierarchical cluster analysis of supervised data (data were filtered based on feature *p* value, $p < 0.05$). The results indicate differential expression patterns between patients and controls (Fig. 1B). Based on visual inspection of the results (Fig. 1A and B), EHS participants with more incidents of heat illness (subject 96 had 4 incidents and subject 100 had 3) were not distinct from participants who had less incidents of heat illness (subject 77 had 1).

3.3. Signaling pathways related to interleukins and cellular stress are down-regulated in EHS participants

To find genes and signaling pathways that might be associated with EHS development, we first assessed average gene expression changes in EHS compared with control participants; next, the top 15% up or down regulated genes in EHS were selected to perform further functional pathway analysis (Fig. 2A). In total, 660 genes were selected (Supplementary Table 1). Using Cytoscape signaling pathway software, we found that genes related to interleukins and cellular stress were significantly inhibited in EHS participants (Fig. 2B and C). For example, the peroxiredoxin (PRDX) gene 3, 5 and 6 all differed significantly between EHS and Controls: for peroxiredoxin 6 the ΔΔEHS Mean was −0.234 as compared to −0.029 for the ΔΔControl Mean (See Supplementary Table 1). Another example is cathepsin S (CTSS) gene: it differed significantly between EHS and Controls (ΔΔEHS Mean = −0.334 and ΔΔControl Mean = 0.046).

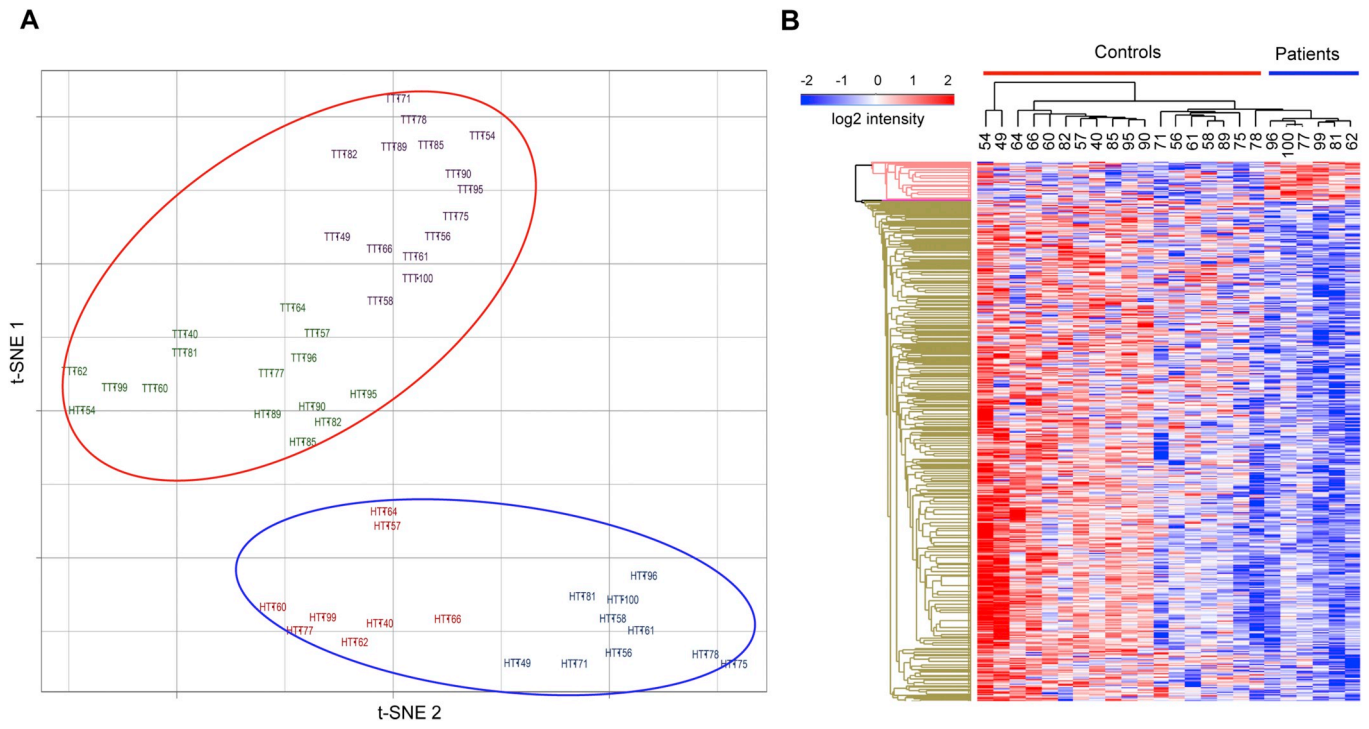

Fig. 1. Gene expression profiling of EHS and Control participants. **A**) t-distributed stochastic neighbor embedding analysis (t-SNE) shows the difference of gene expression pattern between ΔHTT (Heat Tolerance Test, blue circle) and ΔTTT (Themal-neutal Tolerance Test, red circle). Different sample colors represent sub-clusters. **B**) Hierarchical cluster analysis of supervised data (filtered at $p < 0.05$) shows differences between EHS ($n = 6$) and control ($n = 18$) participants.

4. Discussion

EHS poses a severe threat to mission-readiness, and individuals who experience one incident are at much higher risk for incidents in the future (Nelson et al., 2018). Epstein (1990) previously provided two possible reasons why some people have a predisposed risk for EHS: 1) they may have an underlying genetic predisposition or 2) damage from an initial EHS may increase their susceptibility to a future incident. Our results support the first reason, but do not necessarily rule out the second. Our current research indicates that a panel of genes related to interleukins and cellular stress are significantly down-regulated in participants with a history of EHS compared to their non-EHS counterparts when exposed to exercise under a high temperature (40 °C).

One important finding of this study is that a panel of genes involved in immune/cellular response to stress was remarkably down-regulated in EHS participants (Fig. 2B). For example, the PRDX genes are part of a family of antioxidant enzymes involved in redox regulation and purportedly control cytokine-induced peroxide levels (Rhee et al., 2005; Sharma et al., 2013). They likely serve a role in protecting against oxidative injury and down regulation would likely compromise protection. Importantly, peroxiredoxin 6 was shown to be associated with the erythrocyte membrane in response to *in vitro* thermal stress (Sharma et al., 2013). Our other example, cathepsin S gene (CTSS), was also markedly transcriptionally downregulated in EHS as compared to controls, and this protein, as a member of the cysteine cathepsin protease family, has been shown to promote degradation of damaged or unwanted proteins in the endo-lysosomal pathway (Wilkinson et al., 2015). In addition it is now being considered as a potential biomarker for various diseases (such as arthritis, cancer, and cardiovascular disease (Liu et al., 2016; Wilkinson et al., 2015)), and for stratifying patients to increase the effectiveness of therapeutic strategies (Fernandez et al., 2001; Wilkinson et al., 2015; Xu et al., 2009).

Activation of cellular stress reactions can physiologically protect tissues/cells from heat stress-induced complications, such as cellular damage and death. Thus, transcriptional suppression of these genes would negatively affect an EHS participant's response to thermal stress (Sonna et al., 2007). For example, IL-6 and TNF double receptor knockout mice showed higher mortality rates than their wild-type controls following heat stroke collapse (Leon et al., 2006). In general, levels of heat shock proteins (HSP) are increased in thermal stress conditions (Lee et al., 2018). However, EHS participants did not exhibit any significant changes in HSP genes, except for up-regulation of HSPA8 gene and down-regulation of HSPA6 genes.

Cytokine and chemokine responses to EHS have been studied in both animals and humans (Heled et al., 2013; King et al., 2017; Li et al., 2018). In most cases these cytokines/chemokines, such as IL-6, IL-10, granulocyte-colony-stimulating factor (G-CSF), macrophage inflammatory protein (MIP)-2, and MIP-1β, increase in response to exertional heat stress, and the magnitude of the response is diminished after heat acclimation (Li et al., 2018). These immune/inflammatory responses are believed to initiate survival pathways and early onset tissue repair mechanisms (Sonna et al., 2007). Indeed, our results support the above observations. When we performed functional enrichment analysis using Metescape on the top 200 down-regulated genes (see detail in Supplementary Table 2) in the EHS group compared with the non-EHS controls, we found a decrease in the functions of cytokine secretion, response to bacterium and cellular response to oxidative stress (Supplementary Figure). This was consistent with results from the signaling pathway analysis (Fig. 2C). Metascape enrichment analysis also showed that leukocyte activation and cellular stress response were attenuated in EHS participants (Supplementary Figure). Thus, suppression of immune/-stress response functions may be one of the mechanisms which makes EHS participants susceptible to thermal stress during exercise.

The HTT is not without its limitations, and there is debate about its clinical utility for identifying patients who are heat intolerant (Mitchell et al., 2019). Within our sample, more non-EHS controls were determined to be heat intolerant that heat patients. Across the two groups, mean final Tr (patients: 38.0 ± 0.29; controls: 37.9 ± 0.29) and HR (patients: 121 ± 18; controls: 125 ± 23) from the HTT were similar, although final HR exceeded the heat tolerance cutoff of 150 for five

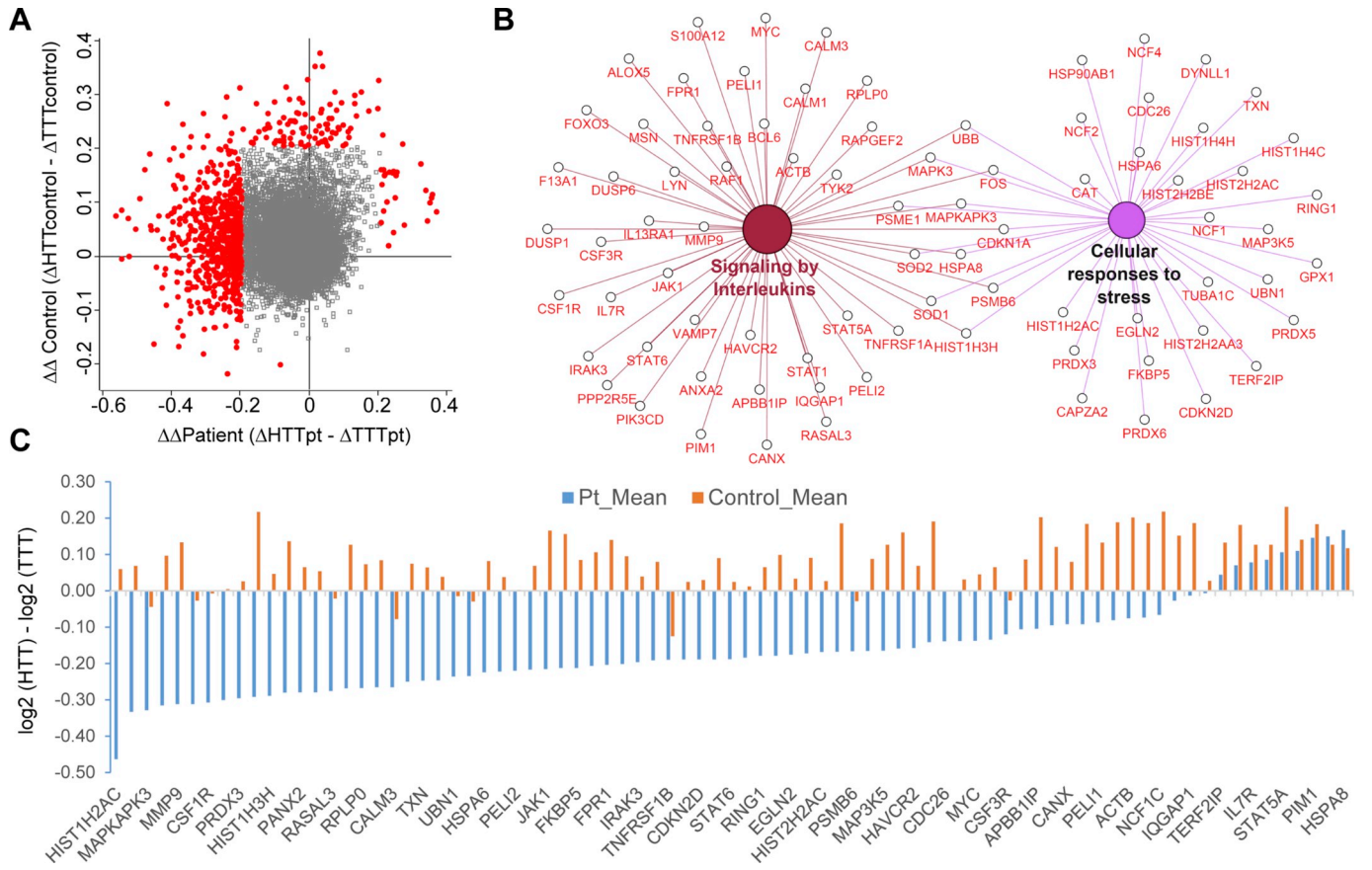

Fig. 2. Enrichment analysis of molecular signatures of average gene expression changes. **A**) Scatterplot of $\Delta\Delta HTT_{EHS}$ versus $\Delta\Delta HTT_{control}$. Red spots represent any data ≥ 0.2 or $\leq$ -0.2, which is approximately equal to ±15% changes. The detailed gene list is provided in Supplementary Table 1. The gray spots represent the remaining data. **B**) Functional networks of genes from above red spots were analyzed using Cytoscape software and generated with ClueGO (cytoscape.org). **C**) The majority of genes related to interleukins and cellular stress are transcriptionally decreased. The gene expression changes were calculated as described in detail in the Materials and Methods.

non-EHS controls. Therefore, this might reflect the cutoffs used for heat tolerance with the HTT (which are also under some debate), or it might reflect the two groups' different backgrounds. Even though we attempted to match both groups based on VO_{2max} and %BF, these measures may have been less valid for the EHS group than for the non-EHS group, because the heat patients tended to be from more physically demanding military occupational specialties than the non-EHS controls, and some may have been on duty-restrictions. Additionally, even though we did not observe any effect of season on heat tolerance, the possible effects of acclimatization cannot be completely ruled out, because many behavioral factors influence it (e.g., activity levels/restrictions, indoor vs. outdoor activity). The overall inferences from our study are also limited by the small sample size, particularly for heat patients. Therefore, a large cohort study would be required to further confirm our results. It would also be more informative to perform global protein profiling of participants' serum/plasma based on mass spectrometer in the future. Moreover, gene expression profiling in combination with proteomics would give us more accurate information with regard to gene/protein changes associated with EHS development. Nonetheless, we believe that findings from our exploratory study provide evidence that more hypothesis-driven research is warranted next.

Taken together, our results demonstrate that 1) both EHS and non-EHS control groups had comparable physiological responses to the HTT and TTT based on maximal Tr and HR parameters. 2) PBMC can provide important insights into human gene expression changes that occur with exercise and heat exposure. The gene expression profile of skeletal muscle tissue might be more relevant to EHS pathophysiology than PBMC, but it is more invasive to collect them in practice, particularly in warfighters. PBMC can be easily obtained from humans and is potentially informative for studying "non-muscle" responses to exertional heat stress. As circulating cells, PBMC are exposed to both systemic and local signals present in perfused tissue during exercise, including temperature, pH, oxygen tension, cytokines, and mechanical stresses. In addition, blood drawn into the PAXgene or Tempus Blood RNA tube is stable for up to 5 days at room temperature, or minimally 7 days at 4 °C, which makes RNA sequencing on blood samples practically applicable. Therefore, gene expression profiling of PBMC should reflect systemic responses to a wide variety of stimuli, in particular exertional heat stress, as shown in different clusters between HTT and TTT conditions (Fig. 1A). 3) Our results, along with other studies' (Bouchama et al., 2017; Sonna et al., 2004), show that the genes related to immune responses and cellular stress are mostly suppressed in participants with a history of EHS compared to their non-EHS counterparts. However, there are two limitations to this study to note. First, our study was not designed to monitor dynamic gene expression changes across different time-points pre- and post-heat tolerance testing. A single time-point gene expression value may not correctly reflect clinical significance. Second, the sample population used for this study was small, particularly given the wide genetic variation between individual subjects. Thus, whether suppression of these genes is associated with susceptibility to exertional heat injury requires further investigation, and this in turn might lead to improvements in the prevention and treatment of EHS in clinic.

Funding

The project was funded by the Office of Naval Research, grant no. N0001411MP20023, USA.

Declaration of competing interest

The authors have no conflicts of interest or financial interests to disclose.

Acknowledgements

The results of the current study to not constitute an endorsement of any product by the authors or the journal. The opinions and assertions expressed herein are those of the author(s) and do not necessarily reflect the official policy or position of the Uniformed Services University, the Department of Defense, or the Henry Jackson Foundation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.102423.

References

Amit, D., Itay, K., Ran, Y., Yoram, E., Heled, Y., 2013. Refining the distinction between heat tolerant and heat intolerant individuals during a heat tolerance test. J. Therm. Biol. 38, 539–542.
Bedno, S.A., Urban, N., Boivin, M.R., Cowan, D.N., 2014. Fitness, obesity and risk of heat illness among army trainees. Occup. Med. 64 (6), 461–467.
Bouchama, A., Aziz, M.A., Mahri, S.A., et al., 2017. A model of exposure to extreme environmental heat uncovers the human transcriptome to heat stress. Sci. Rep. 7 (1), 9429.
Bouchama, A., Knochel, J.P., 2002. Heat stroke. N. Engl. J. Med. 346 (25), 1978–1988.
Epstein, Y., 1990. Heat intolerance: predisposing factor or residual injury? Med. Sci. Sport. Exerc. 22 (1), 29–35.
Epstein, Y., Druyan, A., Heled, Y., 2012. Heat injury prevention-a military perspective. J. Strength Cond. Res. 26 (Suppl. 2), S82–S86.
Fernandez, P.L., Farre, X., Nadal, A., et al., 2001. Expression of cathepsins B and S in the progression of prostate carcinoma. Int. J. Cancer 95 (1), 51–55.
Heled, Y., Fleischmann, C., Epstein, Y., 2013. Cytokines and their role in hyperthermia and heat stroke. J. Basic Clin. Physiol. Pharmacol. 24 (2), 85–96.
Heled, Y., Moran, D.S., Mendel, L., Laor, A., Pras, E., Shapiro, Y., 2004. Human ACE I/polymorphism is associated with individual differences in exercise heat tolerance. J. Appl. Physiol. 97 (1), 72–76.
Kazman, J.B., Heled, Y., Lisman, P.J., Druyan, A., Deuster, P.A., O'Connor, F.G., 2013. Exertional heat illness: the role of heat tolerance testing. Curr. Sports Med. Rep. 12 (2), 101–105.
King, M.A., Leon, L.R., Morse, D.A., Clanton, T.L., 2017. Unique cytokine and chemokine responses to exertional heat stroke in mice. J. Appl. Physiol. 122 (2), 296–306.
Kyle, S.B., Smoak, B.L., Douglass, L.W., Deuster, P.A., 1989. Variability of responses across training levels to maximal treadmill exercise. J. Appl. Physiol. 67 (1), 160–165.
Lee, E.C., Leon, L.R., Adams, W.B., Arent, S.M., Maresh, C.M., Walth, N.P., 2018. Biomarkers. In: Casa, D.J. (Ed.), Sport and Physical Activity in the Heat. Springer International Publishing, New York, NY, pp. 191–212.
Leon, L.R., Blaha, M.D., DuBose, D.A., 2006. Time course of cytokine, corticosterone, and tissue injury responses in mice during heat strain recovery. J. Appl. Physiol. 100 (4), 1400–1409.
Leon, L.R., Helwig, B.G., 2010. Heat stroke: role of the systemic inflammatory response. J. Appl. Physiol. 109 (6), 1980–1988.
Li, Q., Sun, R., Liu, S., et al., 2018. Effect of heat acclimatization training on inflammatory reaction and multiple organ dysfunction syndrome in patients with exertional heat stroke. Zhonghua wei zhong bing ji jiu yi xue 30 (6), 599–602.
Li, Y., Wang, Y., Ma, L., 2014. An association study of CASQ1 gene polymorphisms and heat stroke. Genom. Proteom. Bioinform. 12 (3), 127–132.
Liu, W.L., Liu, D., Cheng, K., et al., 2016. Evaluating the diagnostic and prognostic value of circulating cathepskin S in gastric cancer. Onctoarget 7 (19), 28124–28138.
Mitchell, K.M., Cheuvront, S.N., King, M.A., et al., 2019. Use of heat tolerance test to assess recovery from exertional heat stroke. Temperature 1–14.
Moran, D.S., Erlich, T., Epstein, Y., 2007. The heat tolerance test: an efficient screening tool for evaluating susceptibility to heat. J. Sport Rehabil. 16 (3), 215–221.
Moran, D.S., Shitzer, A., Pandolf, K.B., 1998. A physiological strain index to evaluate heat stress. Am. J. Physiol. 275 (1 Pt 2), R129–R134.
Nelson, D.A., Deuster, P.A., O'Connor, F.G., Kurina, L.M., 2018. Timing and predictors of mild and severe heat illness among new military enlistees. Med. Sci. Sport. Exerc. 50 (8), 1603–1612.
O'Connor, F.G., Casa, D.J., Bergeron, M.F., et al., 2010. American College of Sports Medicine Roundtable on exertional heat stroke–return to duty/return to play: conference proceedings. Curr. Sports Med. Rep. 9 (5), 314–321.
Ogawa, H., Fujitani, K., Tsujinaka, T., et al., 2011. InBody 720 as a new method of evaluating visceral obesity. Hepato-Gastroenterology 58 (105), 42–44.
Phillips, N.A., Welc, S.S., Wallet, S.M., King, M.A., Clanton, T.L., 2015. Protection of intestinal injury during heat stroke in mice by interleukin-6 pretreatment. J. Physiol. 593 (3), 739–753.
Rhee, S.G., Chae, H.Z., Kim, K., 2005. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. Free Radic. Biol. Med. 38 (12), 1543–1552.
Sharma, S., Zingde, S.M., Gokhale, S.M., 2013. Identification of human erythrocyte cytosolic proteins associated with plasma membrane during thermal stress. J. Membr. Biol. 246 (8), 591–607.
Shibolet, S., Lancaster, M.C., Danon, Y., 1976. Heat stroke: a review. Aviat. Space Environ. Med. 47 (3), 280–301.
Sonna, L.A., Sawka, M.N., Lilly, C.M., 2007. Exertional heat illness and human gene expression. Prog. Brain Res. 162, 321–346.
Sonna, L.A., Wenger, C.B., Flinn, S., et al., 2004. Exertional heat injury and gene expression changes: a DNA microarray analysis study. J. Appl. Physiol. 96 (5), 1945–1953.
Stearns, R.L., Deuster, P., Kazman, J.B., Heled, Y., O'Connor, F.G., 2018. Heat tolerance testing. In: Casa, D.J. (Ed.), Sport and Physical Activity in the Heat. Springer International Publishing, New York, NY, pp. 213–227.
van der Maaten, L., Hinton, G., 2008. Visualizing data using t-SNE. J. Mach. Learn 9, 2579–2605.
Wilkinson, R.D., Williams, R., Scott, C.J., Burden, R.E., 2015. Cathepsin S: therapeutic, diagnostic, and prognostic potential. Biol. Chem. 396 (8), 867–882.
Xu, J., Li, D., Ke, Z., Liu, R., Maubach, G., Zhuo, L., 2009. Cathepsin S is aberrantly overexpressed in human hepatocellular carcinoma. Mol. Med. Rep. 2 (5), 713–718.
Yu, T., Dohl, J., Chen, Y., Gasier, H.G., Deuster, P.A., 2019. Astaxanthin but not quercetin preserves mitochondrial integrity and function, ameliorates oxidative stress, and reduces heat-induced skeletal muscle injury. J. Cell. Physiol. (ahead of print).
Yu, T., Ferdjallah, I., Elenberg, F., Chen, S.K., Deuster, P., Chen, Y., 2018. Mitochondrial fission contributes to heat-induced oxidative stress in skeletal muscle but not hyperthermia in mice. Life Sci. 200, 6–14.