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Structural Variations in Circulating Lipopolysaccharide may Increase Severity of Exercise-Induced Heat Illness

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Structural variations in circulating lipopolysaccharide may increase severity of exercise-induced heat illness

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ABSTRACT

Previous investigations at the Falmouth Road Race (FRR; Falmouth, MA) suggest that combined exercise, heat, and dehydration stress increase circulating lipopolysaccharide, associated binding proteins, and downstream immune signaling. Lipopolysaccharide (LPS), a component of gram-negative bacterial membranes, may be released into circulation from the gut with increased gastrointestinal permeability observed post-exercise. Circulating LPS activates host immune cell signaling that can lead to inflammatory responses that upregulate pro-inflammatory gene expression (eg TNF-α, IL-1β), which if unchecked, may lead to pathophysiology (eg cell pyroptosis, exertional heat stroke (EHS), and sepsis). While subjects of the 2015 FRR (n=30) had elevated core temperature post race (39.87°C) compared to pre (36.87°C, p<0.05), there is unexplained variation in the relationship among the level of circulating LPS (2.90±2.6EU/ml pre vs. 3.48±4.0EU/ml post, p<0.05; Hycult Biotech, ELISA), core temperature, and diagnosis of EHS or exertional heat illness. Since some strains of bacteria induce a strong immune response while others do not, we aimed to determine if structural variations in LPS contribute to varying immune system activation during exercise-heat stress. We utilized extraction of LPS from pre-race and post-race plasma and FTIR spectroscopy to characterize structural variations in LPS in individuals of the 2015 and 2017 FRR, and conclude that other methods of analysis may be appropriate to fully understanding this relationship. Acknowledging the diversity of circulating LPS will be critical to answering fundamental questions about EHS pathophysiology and the interaction between gut health and exercise in stressful environments, as this approach has never been taken to examine whether structural variants may explain some of the variability in literature around LPS post exercise and as a potential biomarker for EHS.
LITERATURE REVIEW

Introduction

Current literature and our unpublished data suggest that exercise, heat, and dehydration stress increase gastrointestinal (GI) tract permeability, exposing the circulatory system to components of the microbiome usually found only in the gut. Lipopolysaccharide (LPS) is one such element leaked into circulation; circulating LPS activates host immune cell signaling cascades that can in extreme circumstances lead to cell pyroptosis, increased core temperature, heatstroke, and septic shock.

I. Lipopolysaccharide characterization

LPS is a component of the outer membranes of gram-negative bacteria that contributes to structural integrity and defense of microorganisms (Qiao, 2014). Under normal conditions of the human gut, LPS is secreted from these membranes without the breakdown of the cell wall via bacterial outer membrane vesicles (Kulp, 2010). When a person is not physiologically stressed (ie the absence of heat or dehydration stress), there is little risk associated with this secretion. Released LPS remains within the gastrointestinal tract and is eventually expelled from the body.

Separation of the GI tract and the bloodstream is maintained via tight junction proteins, mucous, chloride, and water secretions at gut epithelial cells (Lakhan, 2010). Under physiological stress, however, the integrity of this barrier can be compromised (Harris, 2012). Paracellular transport is the process by which LPS leaves the colon and enters the bloodstream through “leaky” tight junctions. LPS may then be responsible for host innate inflammatory responses (Harris, 2012).
i. Structural properties

LPS consists of two main components: a lipid and a polysaccharide. The polysaccharide portion is composed of two units: the O antigen and the core polysaccharides. The O-specific hydrophilic side chain has repeating oligosaccharide units that are specific to the bacterial species from which the molecule comes. This antigen portion is highly variable. The inner heptose and outer hexose polysaccharide cores are widely conserved across gram-negative bacteria and are attached to phosphate groups that act to stabilize the molecular structure. While the polysaccharide portion may not be present in all LPS variants, the hydrophobic lipid portion, termed lipid A, must be present for bacterial viability. Lipid A is responsible for the endotoxic effects of LPS (Rietschel, 1994). Variations in the structure of LPS can change the biological activity of the molecule and subsequent immune activation (Schletter, 1995). Because of these structural variations in LPS across bacteria, the molecular weight of the molecule ranges from 10 to 20 kDa (Rietschel, 1994).

![Figure 1. General structure of LPS (Qiao, 2014)](image)

ii. Biochemical pathway

LPS activates myeloid lineage immune cells (specifically circulating macrophages) by a series of molecular interactions and pathways (Liu, 2007). The lipid A portion of LPS acts as a pathogen-associated molecular pattern that is recognized by LPS-binding protein (LBP), a
circulating acute-phase protein. LBP associates with LPS, and then the extracellular membrane bound protein CD14 recognizes the complex and presents it to MD-2. MD-2 is an extracellular adaptor protein that associates with Toll-like receptor 4 (TLR4). This association triggers homodimerization of TLR4 and subsequent signal transduction through intracellular cascades. TLR4 is a major component in the recognition of the molecular patterns associated with bacterial infections and specifically LPS by T cells (Liu, 2007). Intracellular Toll-interleukin-1 receptor (TIR) domains allow the TLR4 oligomer to recruit downstream adaptors, including MyD88, TIRAP, TRIF, TRAM and SARM (Pallson-McDermott, 2004).

![Diagram of LPS signaling pathway](image)

**Figure 2.** Representation of LPS signaling pathway (Lu, 2008)
The entire complex of LPS-associated extracellular and intracellular proteins can utilize two separate pathways that have distinct outcomes for the cell: MyD88-dependent and MyD88-independent (Chow, 1999). The MyD88-dependent pathway recruits death-domain containing IRAK molecules and TRAF6 adaptor proteins. Through a series of downstream phosphorylation events, NF-κB is activated. Ultimately, this pathway leads to the release of pro-inflammatory cytokines (e.g., TNF-α and IL-6) from immune cells that promote localized or systemic inflammation (Frost, 2002). The MyD88-independent pathway depends on TRIF adaptor proteins. TRIF recruits TRAF3, another adaptor protein, which activates IRF3 and NF-κB to induce Type 1 interferons (Lu, 2008). These two activation pathways have different consequences for immune cells and are dependent on the shape of the presented lipid (Rietschel, 1994; Lu, 2008).

**iii. Immune system activation**

Structural variations of LPS may lead to differential innate immune responses (Alexander, 2001). Pro-inflammatory cytokines, resulting from the MyD88-dependent TLR4 pathway, can cause infected cells to swell, burst, and die. This form of cell death, pyroptosis, is a noncanonical response, which is dependent on the source bacteria of the LPS (Jorgensen, 2015). For example, *Helicobacter pylori* LPS stimulates the response poorly while *Escherichia coli* LPS produces a robust immune response (Kayagaki, 2013). While this pathway is important to an effective immune response to LPS, too much activation can lead to host inflammation. Similarly, Type 1 interferon, released upon activation of the MyD88-independent TLR4 pathway, is necessary for activating natural killer cells that destroy infected cells (Shi, 2014; Smith, 2015). In general, over-activation of the immune system can be harmful to the host. These responses
include but are not limited to inflammation, increased core temperature and fever, heatstroke, and endotoxic shock (Hagar, 2013).

iv. Pro-inflammatory vs. anti-inflammatory responses

The best-characterized responses to LPS in circulation are pro-inflammatory. The subsequent release of pro-inflammatory cytokines and stress hormones may result in fatigue, mood changes, and underperformance (Clark, 2016). LPS can also activate circulating macrophages, cause gene expression changes, and lead to more serious pathologies such as endotoxemic shock, especially in people who consume high plant-based diets (Clark, 2016; Aung, 2005).

Anti-inflammatory LPS responses to exercise have been less well characterized and primarily in murine models. In vitro, LPS added exogenously to cultured macrophages leads to a robust immune response, including B cell and macrophage activation. However, when LPS is injected into live mice, an anti-inflammatory response is described. The response includes a decreased accumulation of macrophages when the injection is paired with another inflammatory stimulus for at least 72 hours, as compared to mice resistant to LPS. In the resistant strains, LPS exacerbates the immune response caused by another inflammatory stimulus (Verghese, 1981). This difference in response may be caused by many factors, including the difference between exogenous and endogenous LPS.

v. Endogenous TLR4 agonists

Although LPS is the major activator of TLR4 on immune cells (e.g., T cells, dendritic cells, macrophages), there are also other agonists that may activate the pro-inflammatory pathway in humans. Some of these include: heat shock proteins 60 and 70 (involved in protein folding and cellular stress resistance), resistin (involved in insulin resistance), and β-defensin (involved in
antimicrobial defense) (Erridge, 2012; Liu, 2007). LPS may not be solely responsible for TLR4-induced inflammation post exercise, but not studies have investigated this possibility.

vi. LPS isolation and structural characterization

LPS can be isolated from blood by introducing a polar solvent that interacts with the polysaccharide region of the molecule (Bernhardt, 1991; Rezania, 2011). In brief, plasma or serum samples, that have been stored at -80°C since, are thawed and then treated with excess methanol. After extraction, the supernatant can be analyzed for structural characterization (Zhao, 2010). Some of the methods for characterizing the molecule include: nuclear magnetic resonance (NMR; Zahringer, 2014), mass spectrometry (Kilar, 2013), chromatography (de Barros, 2015), crystallography (Ferguson, 2000), and infrared spectroscopy (Brandenburg, 1993; Kim, 2005).

Chromatography: There are several chromatographic methods that can be used to resolve molecular structure, including gel filtration and affinity chromatography. In general, chromatography separates proteins based on affinity to the solution they are dissolved in and a stationary phase. Unique variants should occupy distinct spaces along a chromatography plate, and reveal how many different structural variants of LPS are in circulation (Lodish, 2000).

Mass spectrometry: Advances in mass spectrometry have made it an increasingly powerful tool to determine the covalent structure of proteins. The protein must be fractionated into ionized species, analyzed to determine the mass-to-charge ratio of each ion, and then compared to known to resolve the amino acid sequence. Structural properties of both pure and mixed solutions are determined by shifts in intensity and positions in the spectra (Domon, 2006).

NMR: NMR can be used to determine two and three dimensional protein structure in a solution. This technique is best suited for proteins between 5 and 25 kDa. NMR measures nuclei
spin in a magnetic field, which is translated into signals that account for chemical shift, folding of a molecule, and the relationship of one atom to its neighbor (Poulsen, 2002).

Crystallography: This method is especially useful for determining the three-dimensional structure of a protein. It requires concentrating and crystallizing the molecule and then using X-rays to create an electron density map. This map can be translated into a resolved structure at the atomic level. Crystallography can also elude to functionality of a molecule (Lodish, 2000).

High performance liquid chromatography mass spectrometry (HPLC-MS): Solutions of the compound of interest are added to a HPLC column packed with silica. Compounds separate based on interactions with the stationary phase (ie the silica) and the mobile phase (ie the solvent eluent). The eluted components are then analyzed with electrospray ionization or atmospheric pressure chemical ionization (Gates, 2018). Extraction of LPS from plasma is necessary to perform this kind of analysis.

Fourier transform infrared spectroscopy (FTIR): FTIR uses vibrational or rotational motions of functional groups on molecules to produce a series of absorbances over a spectrum of wavelengths. Functional group peaks are assigned to specific regions on the molecule according (Kim, 2004). Extraction of LPS from plasma is necessary to perform this kind of analysis.

**Figure 3 (from Kim, 2005).** Example of the output of FTIR from isolated LPS of *Salmonella enterica* serotypes. The numerical labels correspond to: fatty acid region (I), amide region (II), mixed region (III), polysaccharide region (IV), and fingerprint region (V).
II. The gut microbiome

The composition of the gut microbiome can indirectly influence immune responses. When the gut becomes leaky in response to increased core temperature, for example, components of the microbiome are released into systemic circulation (Sekirov, 2010). These components in circulation can then lead to differential pathophysiology, and we hypothesize, during or after exercise.

i. Characterization of the microbiome

Under normal conditions, the gut is stably separated from its external environment by an intestinal barrier that is both physical and chemical. The physical barrier is cellular, and consists of vascular endothelium, epithelium, and mucosal cells. The chemical barrier consists of immune cells, digestive secretions, and cellular secretions such as cytokines and inflammatory mediators. Together these two layers help maintain balance for the body. They prevent water and electrolyte loss, allow nutrient absorption, and play an important immune function by preventing pathogens from entering the body through the gut. When the normal flux of molecules in and out of the gut is not functioning properly in equilibrium, systemic problems can arise, including metabolic diseases, neuropathologies, and sepsis (Bischoff, 2014).

Intestinal microbiota, normally contained within the gut, are mostly symbiotic with the host. Bacterial colonies in the gut tend to be very dense, up to $10^{12}$ bacteria per gram of intestinal luminal content. Individual guts typically have several hundred species of bacteria from two major phyla, with *Bacteroidetes* and *Firmicutes* making up 99% of the human gut (David, 2014). The microbiome plays a role in nutrient breakdown and absorption, the prevention of colonization by harmful pathogens, and vitamin production. This bacterial layer provides a third line of defense in the regulation of gut permeability (Fujimura, 2010).
ii. Dietary influences on the composition of the microbiome

The exact composition of the gut microbiome is largely dependent on diet. Of the two dominant bacterial phyla in the gut, those from *Bacteroidetes* become more prevalent in the gut in an individual who eats an animal-based diet because these bacteria are able to break down protein. In contrast, bacteria from the phyla *Firmicutes*, which are better suited to plant-breakdown, dominate when an individual consumes a strictly plant-based diet. It has been known for some time that the composition of the microbiome can change on a long-term scale, but more recently, it has been demonstrated that due to diet, there can be dramatic, short-term changes that reflect a trade-off between protein and sugar fermentation. Taxonomic shifts can be observed in as few as 3 days after diet modification (Heiman, 2016). Changes in diet not only influence the community structures of microorganisms in the gut, but can also change the gene expression patterns of the microbes (David, 2014; Lloyd-Price, 2016).

iii. Role in immune responses

Much like LPS in circulation can activate immune cascades, intestinal bacteria can activate inflammasomes and proinflammatory cytokines within the gut. These cascade products are necessary for the immune system because they defend the host from intestinal pathogens. This has been demonstrated in germ-free mice through the use of antibiotics: when antibiotics obstruct the microbiome of the host, fewer inflammasomes are produced in response to pathogens and so the host is more likely to develop metabolic pathologies (Round, 2009). Furthermore, disruptions to the normal functioning of the microbiome can lead to autoimmunity, allergies, and cancer because the bacterial colonies of the gut play a large role in the development of the adaptive immune system which normally defends the body from such diseases (Sekirov, 2010; Fujimura, 2010).
The composition of the microbiome is constantly changing, which suggests a reason for why people are better able to fight infections at certain points in their lives (Chen, 2016). Specific key species of microbiota elicit responses necessary for overall health and homeostasis. Without those key species, as a result of diet, drug or alcohol use, as well as other environmental factors, people become vulnerable to infections and diseases (Bischoff, 2014). The role of the microbiome in systemic immune responses is not completely understood, but it is clear healthy and diseased states in mammals are largely influenced by the gut’s microbial composition.

iv. Microbiome changes as a function of aging

Aging is associated with changes to the gut microbiome in terms of composition and stability. Specifically, it has been reported that aging comes with enrichment in Proteobacteria, a group containing bacteria that are known to cause pathology. It has also been noted that an aged microbiome is associated with a decrease in genes for short-chain fatty acid production and loss of saccharolytic potential, in addition to changes in circulating inflammatory cytokines (eg IL-6 and IL-8). In people over 70 years old, there is loss of microbial diversity, but there is little predictive power for these changes because of other systemic age associated changes, including changes in diet and physical activity and an overall increase in chronic inflammation (Buford, 2017).

Some of the changes to the microbiome associated with aging are related to a typical western diet, which tends to be rich in high saturated fats and sugars. Over time, too much fat and sugar lead to depletion of microbial diversity and an increase in chronic inflammatory disorders (Buford, 2017). In addition, nutrient absorption decreases with deterioration of salivary function and digestion. Taken with chronic activation of the immune system, the gut becomes especially vulnerable to rapid composition changes with increasing age (Buford, 2017).
III. Exercise in the heat

Exercise in stressful environments and under conditions, like hypohydration, that increase physiological stress, impacts gut physiology. In part due to induction of gut leakiness, exercise in the heat can lead to an unsafely elevated core temperature, heatstroke, and septic shock. While altering the composition of the gut microbiome may be able to mitigate some of the risk of these extreme pathologies, there are currently few recommendations in the literature on how to accomplish this and whether this is even a possibility.

i. Exercise as an inducer of gut leakiness

Under non-stressful conditions, the gut is isolated from the rest of the body by tight junctions between intestinal epithelium cells (Lamprecht, 2012). There are many factors that can alter the integrity of these tight junctions, such as taking aspirin. While the barrier is typically tightly regulated, it does not take much to alter the balance and increase permeability to the outside environment (Farhadi, 2008). A leaky gut is also thought to be responsible for several autoimmune diseases, such as rheumatoid arthritis and inflammatory bowel disease, and central nervous system dysfunctions including anxiety and depression (Julio-Pieper, 2016). When the body is exposed to high levels of inflammatory elements from the gut, there can be a systemic response, as evidenced by the occurrence of seemingly unrelated diseases.

Research suggests that exercise, particularly in heat, can lead to an increase in gut leakiness (Lamprecht, 2012). Athletes who exercise strenuously are susceptible to cramps, blood in the stool, nausea, and bloating because of decreased tight junction functioning (Lamprecht, 2012). This causes immune cells to be exposed to pathogens normally contained within the gut, and thus an immune response to be induced (Lamprecht, 2012). While intense exercise can be
harmful, it is worth noting that moderate exercise can have an anti-inflammatory response, especially in aged people (Karper, 2011).

ii. Immune system activation

Exercise in the heat can negatively affect exercise performance by impacting endurance and metabolic and/or cardiovascular responses (Vargas, 2016). With increasing core temperature, the autonomic nervous system responds by diverting blood from the core and toward peripheral circulation to increase evaporative cooling (Sawka, 1993). This diversion is associated with increases in GI leakiness and subsequent presence of LPS in circulation post-exercise as the intestinal barrier dysfunctions (Lambert, 2008). If too much LPS enters systemic circulation, there is potential for the development of sepsis, heat stroke, and even death (Bosenberg, 1985). For most people, there is only a mild immune response to elevated LPS, but depending on the type, intensity, and duration of exercise, a prolonged inflammatory response may be elicited and result in these pathophysiologies (Ziemann, 2013).

iii. Long and short term effects

In the short-term, LPS induces an acute phase inflammatory response in individuals who exercise in the heat. The response is varied, however. In some, core temperature remains in a safe range and there is no central nervous system dysfunction. Others may develop exertional heat illnesses after engaging in the same type and intensity of exercise. The expression of protein biomarkers for exercise-induced heat stress (eg interleukin-6, heat shock protein 72, immunoglobulin M) may become elevated during exercise and for a short amount of time post exercise (Barberio, 2015).

Acclimation to exercise in hot and humid environments may reduce intestinal permeability and minimize the presence of LPS in circulation (Guy, 2016). Additionally, the
extent to which biomarkers of heat illness are expressed can be reduced with training (Barberio, 2015). The LPS self-immunization theory suggests that repeated bouts of exercise in the heat can have protective effects. People with chronic low-grade LPS in circulation can develop a tolerance to LPS and thus to exercise-heat stress (Armstrong, 2018). Alternately, others with chronic LPS in circulation may develop pathologies, including diabetes, kidney disease, and obesity (Glaros, 2013; McIntyre, 2010). It is unclear where the distinction lies between protective and detrimental effects of chronic LPS in circulation.

**Conclusion**

Exercise in the heat can lead to a wide variety of systemic responses. These reactions are influenced largely by an individual’s fitness level, gut microbiome composition, and pre-existing conditions. When the gut becomes leaky post-exercise in the heat, LPS is released into circulation. While low levels of LPS are not necessarily harmful, elevated concentrations in the blood can lead to extreme pathologies like septic shock. It has been suggested in the literature that the source bacteria from which the LPS came can determine the severity of the immune response, which leads to the conclusion that there must be structural variations that account for these differences. Determining exactly what the structural variations exist will elucidate how LPS responds to exercise-heat stress.
METHODS

Subjects

Participants of Falmouth Road Race (2017) were recruited to assess and identify genetic and cellular risk factors for exertional heat illness during summer road races. 32 participants (19 males; age, 45.03±11.62 \(\text{y}\); body mass, 73.95±13.65 kg; height, 174.78±9.21 cm; maximal aerobic capacity, 40.45±6.81 ml•kg\(^{-1}\)•min\(^{-1}\)) consented to participate. Study inclusion criteria included: no chronic health problems, no history of cardiovascular, metabolic or respiratory disease, no fever or other illness at the time of the race, no gastrointestinal motility disorders, no experience of syncope during exercise, no family history of malignant hypothermia, no problems with anesthesia, and predicted to finish the race in under 60 minutes. All subjects were briefed and provided informed consent. This study was approved by the University of Connecticut Institutional Review Board.

Between late July and early August 2017, participants were asked to visit the Human Performance Laboratory at the University of Connecticut to complete a maximum oxygen consumption test (VO\(_{2}\)\(_{\text{max}}\)) and a 7.1-mile run test. Body composition, height, body weight, heart rate, urine specific gravity and urine color, and rectal temperature were measured during this visit. On the night before the race, participants ingested a gastrointestinal temperature pill to monitor core temperature via a CoreTemp ELITE device attached to a waist strap during the race.

Race day measures and analysis

On race day (August 20, 2017), subjects reported to a research tent for pre-race and post-race: physiological measurements (eg body mass, urine specific gravity), and blood and urine samples collection. Urine was analyzed for specific gravity and color on site and discarded.
mL of whole blood was collected from the antecubital fossa veins into serum, EDTA-plasma, and sodium-heparinized cell preparation plasma (CPT) vacutainers by trained phlebotomists. Vacutainers were centrifuged for 15 minutes at 1800 g and 4°C (Eppendorf, Centrifuge 5810 R, 15-amp version, Hamburg, Germany) and aliquoted on site.

EDTA and sodium-heparinized plasma samples were stored in 1 mL aliquots on dry ice and then transferred to a -80°C freezer (-80°C Low Temperature Freezer, Thermo Electron Corporation, Model 8607, Marietta, OH) within 18 hours of collection. Peripheral blood mononuclear cells (PBMCs) were aliquoted from CPT vacutainers and stained with fluor-conjugated antibodies on site. In brief, PBMCs were centrifuged at 200 g for 5 minutes at 4°C, washed and fixed in 1x phosphate buffered saline (PBS)/0.5% bovine serum albumin (BSA)/1.6% paraformaldehyde, and then stained with 1x PBS/0.5% BSA and CD14-FITC, CD16-PECy7, and CD284-PE antibodies for 30 minutes. After a final wash in 1x PBS/0.5% BSA, cells were resuspended in 1x PBS/0.5% BSA, and analyzed via flow cytometry (Accuri C6 Flow Cytometer, C Sampler) on site within 12 hours of staining.

Biochemical analyses

LPS and LPS-binding protein (LBP) concentrations in EDTA-plasma samples were determined in duplicate by ELISA (Hycult Biotech, Uden, The Netherlands). LPS samples were heat-shocked at 37°C for 5 minutes to eliminate endotoxin-inactivating factors and were incubated at room temperature for at least 30 minutes prior to absorbance measurements. LBP samples were diluted 500-fold according to kit recommendations. Both LPS and LBP absorbance measurements were determined on a Versamax tunable microplate reader (Molecular Devices, Sunnyvale, CA) at the wavelength indicated by each kit. Circulating inflammatory cytokines (IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70) concentrations were measured using a cytometric
bead array (BD Biosciences, San Jose, CA, USA) with EDTA-plasma samples and analyzed via flow cytometry. Concentrations of heat shock proteins 90, 60, 27, and 70 in plasma were measured in duplicate by ELISA (StressMarq Biosciences, Victoria, British Columbia) according to kit instructions.

For structural variant determination, LPS was isolated from EDTA-plasma samples and analyzed via attenuated total reflection-Fourier transform infrared spectroscopy (FTIR; Nicolet Magna IR-560 with OMNIC software, Nicolet Instrument Corporation, Madison, WI) and high performance liquid chromatography/mass spectrometry (Synapt G2-Si, Waters Corporation, Milford, MA). For isolation of LPS from plasma, the “methanol method” was used (Zhao, 2010). Samples were added to 100% methanol, vortexed, and incubated on ice for 10 minutes. After centrifugation (5 minutes, 10,000 g, room temperature), supernatant was transferred to a new tube and kept on ice until FTIR analysis.

FTIR analysis was optimized by varying concentration of plasma to methanol (1:0 (a), 1:5 (b), 1:10 (c), 1:15 (d)) to determine which resulted in the least solvent interference (Figure 4). Based on the scans, 1:5 dilution was used for further analysis. Background was collected before each run and 10 µL of sample was added to the instrument for analysis. 128 scans were performed at 4.000 cm\(^{-1}\) with units of absorbance over the spectrum 4000-400 cm\(^{-1}\). Analysis of structural variance was performed by isolating the peak(s) in the spectra corresponding to the polysaccharide region of LPS, between \(~1070\) cm\(^{-1}\) and \(~1170\) cm\(^{-1}\). Exact regions were isolated by points of inflection in the curves using CSV output from the FTIR instrument. Areas under the curve approximations were determined by summing partial squares over the entire relevant wavelength for the plots wavelength vs. absorbance. Linear regressions models were created for pre and post area under the relevant curve vs. post race core temperature of each participant.
Statistical analysis

All data were analyzed using SAS version 9.4 (SAS Institute, Inc., USA). Two tailed paired t-tests were used for pre and post race comparisons of USG, core temperature, body mass, TLR4 MFI on CD14 and CD16 cells, circulating [LBP], circulating [LPS], and circulating [HSP60]. Linear regression analysis was used to determine the relationship between FTIR absorbance values in the polysaccharide region of the spectra and post race core temperature. Significance level was set at p<0.05 for all variables and comparisons, and all data are presented as mean ± standard deviation.
RESULTS

Participants

32 participants (19 males; age, 45.03±11.62 yrs; body mass, 73.95±13.65 kg; height, 174.78±9.21 cm; maximal aerobic capacity (VO$_{2\text{max}}$), 40.45±6.81 ml/kg/min) completed baseline and race day measures and testing at the 2017 Falmouth Road Race. VO$_{2\text{max}}$ was assessed within 1 month of race day (July 23, 2017-August 5, 2017) to establish baselines aerobic capacity of all participants. Pre-race minimal clothing body mass, urine specific gravity (USG), and blood draws were completed within 4 hours of the start of the race (collection occurred 5:00-7:00 am; race began at 9:00 am). Post-race minimal clothing body mass, USG, and blood draws were completed within 1 hour of each subject’s individual finish time (course closed by race officials at 11:30 am). Core body temperature was continuously monitored during the race via an ingestible thermistor. Significant differences (p<0.05) were found in core temperature (elevated post race) and body mass (decreased post race) but not in USG, suggesting dehydration stress was mitigated by pre-race euhydration or ad libitum fluid intake during the race (Table 1).

TLR4 activation, LPS, and LBP activity

Data are presented for 2015 FRR and FRR 2017 for comparison across the years due to differences in environmental conditions (temperature$_{2015}$, 84°F; temperature$_{2017}$, 72°F). TLR4 activation was measured via fluor-conjugated antibody staining on CD14+ cells and CD16+ cells. No significant difference (p>0.05) pre vs. post of mean fluorescence intensity (MFI) of TLR4 expression on CD14+ cells for 2017 subjects, but a significant increase in MFI was observed in 2015 subjects in post and recovery (30-60 minutes after finishing the race) samples (Figure 5). Significant increase (p<0.05) pre vs. post of mean fluorescence intensity of TLR4 expression was observed on CD16+ cells in 2017 and 2015. MFI also significantly increased vs.
pre in recovery samples in 2015 (Figure 6). Concentration of circulating LPS binding protein was not significantly different (p>0.05) pre vs. post in 2015 or 2017 samples, or in recovery samples in 2015 (Figure 7). Concentration of circulating plasma LPS was not significantly different (p>0.05) pre vs. post in 2017. In 2015, however, significant increases in circulating LPS in pre vs. post and recovery samples were measured (Figure 8). Concentration of circulating HSP 60, an endogenous ligand of TLR4, was not significantly altered pre vs. post race in 2017 (Figure 9).

LPS structural analyses

Resolution was not high enough to elucidate structural variation in the polysaccharide region of LPS using only 32 scans on the FTIR instrument (Figure 10). Therefore, 128 scans were used for all data collection, as it was possible to visualize and quantify shifts in absorbance peaks at that higher resolution (Figure 11). Linear regression analysis of FTIR spectra for pre and post-race samples (FRR 2015 and FRR 2017) revealed no significant correlations between LPS variants and post-race core temperature (Figure 12, Table 2).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean (±SD)</th>
<th>Significantly different pre vs. post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>45.03 (11.62)</td>
<td>N/A</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>174.78 (9.21)</td>
<td>N/A</td>
</tr>
<tr>
<td>VO_{2max} (ml/kg/min)</td>
<td>40.45 (6.81)</td>
<td>N/A</td>
</tr>
<tr>
<td>VO_{2max} heart rate (bpm)</td>
<td>170.71 (9.56)</td>
<td>N/A</td>
</tr>
<tr>
<td>USG pre</td>
<td>1.015 (0.007)</td>
<td>N/A</td>
</tr>
<tr>
<td>USG post</td>
<td>1.013 (0.016)</td>
<td>No, p=0.53</td>
</tr>
<tr>
<td>Core temperature pre (°C)</td>
<td>37.13 (0.534)</td>
<td>N/A</td>
</tr>
<tr>
<td>Core temperature post (°C)</td>
<td>39.44 (0.904)</td>
<td>Yes, p&lt;0.001</td>
</tr>
<tr>
<td>Pre-race body mass (kg)</td>
<td>73.7 (14.0)</td>
<td>N/A</td>
</tr>
<tr>
<td>Post-race body mass (kg)</td>
<td>72.4 (13.7)</td>
<td>Yes, p&lt;0.001</td>
</tr>
</tbody>
</table>

Table 1. Baseline and race-day variables for 2017 FRR participants
Figure 5. TLR4 MFI on CD14+ cells
Figure 6. TLR4 MFI on CD16+ cells
Figure 7. Concentration of circulating LBP
Figure 8. Concentration of circulating LPS
**Figure 9.** Concentration of circulating HSP60

**Figure 10.** Example of post sample FTIR spectra with 32 scans
Figure 11. Examples of pre and post FTIR spectra with 128 scans
### Analysis of Variance

| Samples   | F value | Pr > F  | Parameter Estimate | t Value | Pr > |t|   | R²       |
|-----------|---------|---------|--------------------|---------|------|----|--------|
| Pre 2015  | 0.10    | 0.7560  | 0.16974            | 0.32    | 0.7560| 0.0084|
| Post 2015 | 0.28    | 0.6042  | 0.29779            | 0.53    | 0.6042| 0.0231|
| Pre 2017  | 1.15    | 0.3086  | -0.5432            | -1.07   | 0.3086| 0.1032|
| Post 2017 | 0.12    | 0.7346  | -0.15891           | -0.35   | 0.7346| 0.0120|

**Figure 12/Table 2.** Linear regression models of pre (top (2015) and bottom (2017) left) or post (top (2015) and bottom (2017) right) FTIR absorbance values vs. post core temperature with table of analysis of variance and parameter estimates for each regression model
DISCUSSION

Differences in the absolute values and levels of significance in measured variables between FRR 2015 and FRR 2017 are likely in part due to environmental conditions. Maximum temperature in 2015 was 84°F with 100% humidity, while it was only 72°F with 100% humidity in 2017. It has been noted in mammals that environmental conditions contribute to the inflammatory response after LPS-challenge. Under hypothermic conditions, activation of innate immune cells is lower than under thermoneutral conditions. Furthermore, the immune response is more increased under hyperthermic environmental conditions compared to thermoneutral conditions (Carroll, 2011). These inconsistencies in environmental temperature across the years suggest a potential explanation for the values we observed. Differences in our subject populations across the years may also be partly responsible for the inconsistency in absolute values of LPS, LBP, and TLR4 expression. Standard values of LPS in circulation pre or post exercise have not been set, to date, because LPS release is dependent on too many factors: the type, intensity, and duration of exercise, environmental conditions, and the level of training of each individual (Barberio, 2015). Thus, it would be worth investigating the training levels across the years to determine if in 2017, the runners were more highly trained, and therefore more resistant to inflammatory responses post-exercise than those in 2015.

Similar to our results, Lichte et al. observed that a low dose of LPS (4 ng·kg⁻¹) does not increase TLR4 expression on CD14+ cells, even if there is an inflammatory response (Lichte, 2013). In FRR 2015, TLR4 expression on CD14+ cells significantly increased pre vs. post, but so did the concentration of circulating LPS. The absolute values of LPS in circulation were also higher in 2015 compared to those in 2017. In FRR 2017, however, LPS in circulation did not significantly increase post race, and neither did TLR4 expression on CD14+ cells. Amounts of
LPS and TLR4 expression on CD14+ cells are highly associated with each other; as one increases in absolute value, so does the other (Lichte, 2013). Expression of TLR4 on CD16+ increased pre vs. post in both 2015 and 2017, however. This is reasonable since CD16+ monocytes are implicated specifically in inflamed states, sepsis, and bacteremia (Nagasawa, 2004). Even very low does of LPS in circulation will increase TLR4 expression on CD16+ cells (Rennert, 2016). Since our participants had undergone a stressful bout of exercise immediately prior to the blood draws, we would expect that CD16+ TLR4 expression would be increased pre to post and to a greater extent than on CD14+ cells.

Circulating LPS binding protein (LBP) remained unchanged pre vs. post exercise in FRR 2015 and FRR 2017. We observed similar results to those reported by McLellan et al. In their exertional heat stress model, they found that in trained individuals, over a range of rectal temperatures between 36.9°C and 39.5°C, there was no change in serum LBP values, even though after 39.0°C, plasma LPS began to significantly increase (McLellan, 2009). Participants in FRR 2015 and FRR 2017 reported being trained to the point where they predicted finishing the 7.1-mile race in less than 60 minutes. It therefore is reasonable that we observed no differences in pre vs. post circulating LBP concentrations in our participants, as they self-reported being trained individuals.

While there are several endogenous ligands of TLR4, it is of note that circulating HSP60 concentrations did not change pre vs. post race. HSP60 is a host-derived damage-associated molecular pattern, released into circulation in response to cellular stress. It is associated with inflammation and may contribute to central nervous system dysfunction that is hallmark of exertional heat stroke when it binds to TLRs (Rosenberger, 2014). Because we observed no change pre vs. post in circulating HSP60, it is likely that the activation of TLR4 was not due to
this specific molecule. We can, therefore, predict the effect of LPS played a role in the inflammatory responses we observe, although there may be other endogenous implicated in these responses (Erridge, 2010).

Our results suggest that FTIR spectroscopy is an appropriate tool to visualize and quantify variations in circulating LPS. However, due to several limitations of the instrument, it is necessary to pursue further structural analysis techniques before drawing any conclusions. Doing so will allow us to quantify relative abundances of each variant and potentially understand the three-dimensional structure of the molecule. As binding of receptors is dependent on specific confirmation of molecules, we should understand the shape of each variant as well to fully understand immune reactions to LPS post-exercise. It may also be useful to collect more direct samples for comparison to the blood samples we have analyzed. While blood is more readily available in a field setting, it would be important to have validation that the LPS in circulation is representative of the types of bacteria found in the gut.
FUTURE DIRECTIONS

The aim of this project was to determine if structural variations in circulating LPS can alter presentations of hyperthermia or heat stroke pathophysiology post race. In order to have predictive power in the future, continuing analysis is necessary to more completely qualify structure and quantify relative concentrations of each LPS variant. These goals will be accomplished in several ways, as outlined below:

1. *Continued FTIR analysis with pre and post overlay of all subjects.* By analyzing all samples in a single spectral overlay, we will be able to directly compare structures for the entire group of interest. We may be able to more accurately detect patterns in variation and confidently quantify functional group variation in the polysaccharide region of LPS. It is also necessary to run replicates of each sample and average the spectra (available in the FTIR software) to ensure changes in room pressure, temperature, or airflow are not contributing to any variation in the output.

2. *HPLC/MS analysis.* Literature reports that this method is a preferred way to quantify relative abundance of each LPS structural variant in plasma samples. Because the concentrations of LPS in each sample is relatively low, especially in the 2017 cohort, it may be necessary to concentration the molecule in the samples, which will require optimization trials before analysis.

3. *Diet composition analysis with correlation to core temperature.* Literature suggests that gut microbial composition rapidly changes with diet. Therefore, what each subject consumed in the three days leading up to the race (data available but not represented in this thesis) may be a factor contributing to post race core temperature and exertional heat stress/stroke presentation. By understanding carbohydrate composition, specifically, we
can make estimates of bacterial species within the gut and better pro- vs. anti-inflammatory LPS in circulation.

4. **Age as a covariate of inflammation.** As age is associated with changes to gut microbiome composition toward pro-inflammatory species, it is necessary to introduce age as a covariate in the analysis. Having this variable will give a more complete understanding to the variations not only in LPS structure but also the inflammatory responses in individuals.

5. **Other endogenous TLR4 ligands.** It is necessary to rule out other endogenous TLR4 ligands as activators of TLR4 and subsequent inflammation presentations. While our goal is to determine if LPS variation contributes to exertional heat stroke pathophysiology, we must be confident that other molecules entirely are not responsible for activating the TLR4 pathway and causing immune reactions including increased core temperature and inflammation post exercise.
CONCLUSION

Structural variations of circulating LPS characterized utilizing FTIR spectroscopy pre or post a 7.1-mile race in a hot and humid environment were not predictive of core temperature post race. There appeared to be changes in the polysaccharide regions between samples of the same subject (pre vs. post) and across all subjects, suggesting that FTIR spectroscopy may be a novel method to analyze LPS in circulation. It is necessary to repeat analysis with other techniques (e.g., HPLC/MS) to quantify relative abundance of each structural variant in circulation. More direct measures, including fecal sample analysis, may also be more appropriate to fully understand the variants of LPS that exist within a runner’s gut. Further investigation of exercise-induce gut permeability and the significance of LPS in post race pathophysiological outcomes is needed to completely understand the mechanisms that underlie exertional heat stress, exertional heat stroke, and exercise-induced endotoxemia.
REFERENCES


42. Liu G, Zhao Y. Toll-like receptors and immune regulation: their direct and indirect modulation on regulatory CD4^+ CD25^+ T cells. Immunology 2007;122(2):149-156.


