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Metabolic profiling of firefighter plasma using 1H NMR platform following curcumin, ketone supplementations and carbohydrate restricted diet benefits for oxidative stress suppression

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Metabolic profiling of firefighter plasma using ^{1}H NMR platform following curcumin, ketone supplementations and carbohydrate restricted diet benefits for oxidative stress suppression

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Occupational health surveys reported that first responders such as firefighters (FF) have some of the highest levels of cardiovascular disease in the nation from poor eating habits and lack of exercise. Three studies were established with goals to identify oxidative stress (OS) biomarkers and improve cardiovascular health for FF including: 1) a 28-day-carbohydrate restricted diet (CRD), 2) a heat-house search and clear protocol in personal protection equipment (PPE) plus curcumin supplementation, and 3) a treadmill exercise protocol in PPE with ketone salt supplementation. During those studies, stored blood plasma subsamples were evaluated for targeted antioxidants or untargeted metabolite concentration fluctuations using ^{1}H NMR. Results from the 28-day-CRD tracked 40 metabolites consistently pre- and post-diet using ^{1}H NMR platform. Of these metabolites' acetone, β-hydroxybutyrate, leucine, and valine significantly upregulated while isoleucine downregulated. The plasma from the curcumin supplementation study contained 34 metabolites that were consistently identified. Lactate significantly upregulated immediate after exercise but returned to pre-exercise levels at 30 min post exercise while all the other metabolites were similar. From the ketone salt study 38 metabolites that were consistently identified from the pre- and post-exercise samples. Mean concentrations of acetone

and β-hydroxybutyrate were significantly upregulated as were leucine and valine pre- and post-exercise while isoleucine downregulated. Lactate increased with ketone salt ingestion post-exercise and up to 30 min post-exercise but returned to normal at 24 h post-exercise compared to pre-exercise levels. Six other metabolites significantly differed in concentrations when compared across sampling times with no discernable impacts to OS or other notable trends. Multivariate analyses using principal components analysis (PCA), partial least squares-discriminant analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) models were not supported using cross-validation for Q^2 coefficients and permutations values at $p \leq 0.05$. From these results no metabolites were shown to support transient OS suppression.

DEDICATION

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CHAPTER I

INTRODUCTION

A firefighters (FF) health survey indicated some of highest levels of obesity and cardiovascular disease (CVD) in the United States (Dobson et al., 2013; Poston et al., 2011, 2012). FF cardiovascular disease was responsible for over 45% of line-of-duty fatalities and morbidities (Smith, 2011; Soteriades et al., 2011) with 42% of the fatalities from sudden cardiac death (SCD; Farioli et al., 2014). Even though firefighting is physically and strenuously demanding due to the emergency nature of the profession, there is a prevalence of low fitness, increased obesity after first five years leading to diabetes, hypertension, and other CVD factors.

In addition to poor health of many FF, physiological effects such as oxidative stress (OS), psychological stress, temperature extremes, lack of physical activity and poor nutrition holistically led to the high morbidity percentages. Data from a survey of FF Body Mass Index (BMI-waist circumference and percent body fat) indicated that a high percentage of FF were either overweight (BMI $\geq$ 25 kg/m^2) or obese (BMI $\geq$ 30 kg/m^2) based on a survey of 478 career and 199 volunteer male FF (Poston et al., 2011). The study further determined that career FF were either overweight or obese at 79.5% or 33.5%, respectively compared to volunteer FF that were overweight at similar levels at 78.4% but had much higher obesity rates at 43.2%. The BMI results exceed the general United States population which ranged at 32% to 35.5% for men and women, respectively (Flegal et al., 2010). Similar findings occurred in previous studies evaluating FF health (Tsismenakis et al., 2009; Yoo & Franke, 2009). During these studies the

FF were never screened for actual factors that cause CVD, or they were unaware of the above-mentioned risk factors such as high blood pressure and lipid levels that can impact CVD. Cardiovascular disease has multiple causes that form a collection of conditions called metabolic syndrome. The complex of factors responsible for CVD can lead to atherosclerosis causing ischemia and heart failure.

Metabolic syndrome or syndrome X is defined as a cluster of risk factors associated with the development of CVD (Waldman et al., 2020). The main factors associated with metabolic syndrome include hyperglycemia, high levels of cholesterol, low-density lipoproteins (LDL), triglycerides, hypertension (high blood pressure), insulin resistance, and increased endothelial dysfunction that are usually associated with central obesity (Alshehri, 2010). Of these it was suggested that common risk factors such as elevated blood pressure, high plasma glucose, and atherogenic dyslipidemia cause prothrombotic (increase blood clotting) and chronic proinflammatory states.

Many of these factors support OS from the formation of greater levels of reactive oxidative species (ROS) which correlated with higher cancer rates. These negative metabolic states can naturally be modified or prevented depending on FF genetics through diet modifications and nutrient supplementation coupled with regular exercise regimes. These health modifications can result in positive health changes reducing the damage from underlying causes (Alshehri, 2010; Grundy et al., 2005; Kaur, 2014). Normal exercise is known to support cellular signaling necessary for muscular adaptations (Powers et al., 2020), and reduced hypertension with increased cellular repair signaling for remodeling of damage cells (Radak et al., 2013). However, alternative strategies to support reductions in chronic OS must be investigated for FF.

Poor nutrition is a critical factor in greater adiposity leading to CVD in FF as reported above. A survey of 689 career FF recorded the types of food and drinks on and off duty they consumed during an 1,879-day period (Day et al., 2017). The survey indicated that most of calories they digested were from turkey, chicken, alcohol, salty snacks, and white flour products. Potatoes were the vegetable listed in FF top 20 calory source. While alcohol used for recreation (mainly of beer) was second largest source of calories and in some cases equaled the amount they obtained from food (Haddock et al., 2012; Haddock et al., 2015; Jahnke et al., 2014). Salty snacks and chips, with almost no nutritional benefit, ranked third with high levels of sodium supporting increased hypertension (Day et al., 2017). The fourth most common foods in the survey were noodles and rice made from white flour as processed foods (Day et al., 2014). White flour type foods consist primarily of starch that metabolize into glucose which is stored in adipose cells, reduces insulin sensitivity, and increases blood sugar levels leading to chronic inflammation. Also, sweet pastries, candies, and sugar-sweetened drinks were a significant part of the FF diet (Day et al., 2014). Career FF were asked about their nutritional habits and health related education. From the group questioned, 71% had no dietary plan and 68% confirmed they did not have adequate dietary support to improved cardiovascular health (Yang et al. 2015).

To overcome the lack of FF cardiovascular health education, several studies were conducted that compared dietary or nutritional supplementations and exercise. The FF participated in three studies that included application of exogenous curcumin or ketone salt supplements and were subjected to a carbohydrate restrictive diet (CRD) prior to conducting specific exercise protocols (McAllister et al., 2018; McAllister et al., 2019; Waldman et al., 2020). The studies reported positive anthropometric benefits with exercise and/or oral nutritional

supplementation and results generally trended towards OS suppression associated with CVD. Details of those studies are reviewed below.

Previous Oxidative Stress Studies Using Firefighters

Three studies from previous FF research at Mississippi State University were conducted including a dietary intervention using a flexible low CRD plan (Waldman et al., 2020), ketone ester (McAllister, 2020), and curcumin (McAllister et al., 2018) exogenous nutritional supplements. The studies included varied nutrients with exercise protocols designed to induce acute stress effects on transient OS while secondarily attempting to directly educate the FF firsthand on ways to enhance their metabolic health. Targeted metabolites from blood plasma collected during these studies showed positive benefits of the interventions especially those associated with OS and improved lipid homeostasis levels. Metabolite fingerprinting platforms could provide additional untargeted biomarkers or metabolic details to support the findings from the three published FF studies.

Blood plasma samples from the three FF studies conducted at MSU remained frozen and stored at -80°C. Analysis of these samples using modern approaches may provide additional indirect or direct OS health information. Therefore, a metabolomic investigation of the FF blood plasma samples was conducted using ^{1}H-NMR to identify and quantify untargeted metabolites. This data may lead to targeted approaches evaluating the identified metabolite intermediates and endpoints of metabolic pathways and associated antioxidants to further understand their roles for improving FF CVD health. The quantification and qualification of metabolite concentration data could compare changes in antioxidant and ROS ratios, with other targeted metabolites associated with cardiovascular health. More recently the ^{1}H NMR platform was identified as an effective tool for obtaining untargeted and targeted metabolic data for further evaluating important

metabolic changed that impact cardiovascular health (Duft et al., 2017). This analytical method is particularly useful in research with liquid samples comparing nutritional diets or oral supplementation with exercise eliciting OS (McAllister et al., 2018, McAllister et al., 2019, Waldman et al., 2020). Therefore, the following study using ^{1}H NMR was conducted using the stored FF blood plasma subsamples from the three studies to determine if untargeted metabolites can provide additional metabolic details related to OS and cardiovascular health.

Objective and Hypotheses

Overall Objective:

Determine physiological responses to stress from exercise, and types of nutrition by monitoring changes in concentrations of untargeted metabolites using ^{1}H-NMR direct profiling of blood plasma collected from previous FF studies (McAllister et al., 2018; McAllister et al. 2019; Waldman et al., 2020).

Main Hypotheses:

H_{A1}: Exercise and/or nutritional supplementation alters the FF metabolism by production of signaling metabolites that ultimately results in changes in FF health.

H_{01}: Exercise and/or nutritional supplementation does not alter the metabolites of FF by production of signaling metabolite chemistry downstream.

Specific Hypothesis:

Curcumin and ketone salt supplementations concurrent with a CRD causes changes in metabolite composition and concentrations thereby reducing inflammation, cellular structure, and mitochondrial function for improved cellular health; to determine if this is true, we use ^{1}H-NMR to identify and quantify the metabolites in blood plasma before and after treatments.

Furthermore, a CRD will reduce concentrations of metabolites associated with negative impacts of OS and inflammation leading to CVD.

Aims/Steps:

1) Obtain NMR spectra from blood plasma collected under baseline conditions and identify peaks corresponding to a suite of metabolites.

2) Obtain NMR spectra from blood plasma collected under treatment conditions and identify peaks corresponding to a suite of metabolites.

3) Identify changes in the metabolites between baseline and treated groups.

4) Correlate observed changes with metabolic pathways to directly or indirectly infer cardiovascular health variations based on metabolites and their concentrations.

CHAPTER II

LITERATURE REVIEW

Metabolic Factors Associated with Atherosclerosis

Atherosclerosis is the leading cause of CVD causing narrowing of arteries and plaque which reduce oxygen deliver from blood to all parts of the body. Metabolic factors associated with plaque formation are from upstream adipokines and other hormones which interactions with macrophages, LDLs of high cholesterol content (~50% total volume), and endothelial cells (Kamari et al., 2007). Furthermore, the endocytosis of oxidized LDLs in macrophages is considered a chronic process causing foam cell formation and arterial intima accumulation resulting in atherosclerosis (Zwaka et al., 2001). Increased levels of LDL's containing high cholesterol levels is a major factor contributing to CVD due to transport of these lipoproteins in blood through the body causing arterial deposition versus high density lipoproteins (HDL) which transport cholesterol (~20% total volume) out of body via the liver. LDLs can be modified in several different ways, but two forms of these lipoproteins are associated with endothelial cell injury during atherogenic process including glycated (gLDL) and oxidized (oxLDL) especially during modifications within the arterial walls (Ross, 1999) and leading to CVD.

Oxidative Stress and Cardiovascular Disease

Cardiovascular disease develops from a complex of multiple metabolic affects with the underlying component causing disease being OS (Gracia et al., 2017; Ji et al., 2016; Powers et al., 2020). The OS causes cellular damage especially when ROS are no longer in a hormesis with

antioxidant-ROS ratio being greater for ROS. This imbalance then allows damaging ROS to form, initiating metabolic downstream signaling causing cellular damage leading to CVD. Thus, OS can be defined as balances between antioxidants, protective mechanism, and radicals from ROS coupled with reactive nitrogen species (RNS; Uttlara et al., 2009). When ROS and RNS are in excess they will suppress important metabolic activities (Costa et al., 2002) impairing metabolic functions leading to CVD (Paravicini & Touyz, 2008).

Exercise for Mitigating OS Associated CVD

Exercise is very important mitigator of CVD and for many other health related issues suggesting that physical activity should be part of our lives (Kullo et al., 2007). As with any form of stress related activity, transient ROS will increase while intensities of the exercises can cause increased inflammation and OS (Ji et al., 2016; Park & Kwak, 2016; Powers et al., 2020). The OS is transient and very important for metabolic signaling to support positive cellular functions (e.g., mitochondrial) and adaptations resulting in muscular remodeling and hypertrophy (Ji et al., 2016; Powers et al., 2020). Eccentric exercise stimulates increased myostatin mRNA expression inhibiting myogenesis but decreased within 24 hours (Koska et al., 2000). This mechanism is believed to be related to modulation of ROS mediated through tumor necrosis factor -α (TNF-α) signaling by transcription factor NF-kB (Sriram et al., 2016). Following an aerobic exercise event and depending upon intensity, numerous other studies have shown that OS stress signaling lead to muscle inflammation and soreness (levels depend upon intensity), muscle damage supporting muscle tissue repair and decreased inflammation under normal beginner's aerobic exercise (Newham et al., 1983; Philippou et al., 2012). As exercise increases with increased neurological and physical experience, chronic inflammation can occur often causing hormeric ratio imbalance of prooxidant versus antioxidants causing chronic

cellular tissue degradation (Ji et al., 2016; Simioni et al., 2018). With increased intensities and durations of training workloads increasing ROS levels causing greater energy production from pathways such as electron transport chain (ETC). The ETC is a main source of ROS from mitochondria and to a lesser degree NADPH oxidase and XO pathways associated with cell membranes (Nunes-Silva & Frieitas-Lima, 2015). The exact mechanisms for this underlying exercise initiated aerobic and anaerobic ROS remains uncertain (He et al., 2016). Example of anaerobic exercise ROS formation is XO activation starts with elevated hypoxanthine following sprints (He et al., 2016; Kang et al., 2009). Main point concerning normal exercise is that ROS formation is transient and other stressors increase ROS damage dependent upon oxidant to antioxidants ratios. With extreme exercise ROS levels can negatively impact cellular health (Ji et al., Powers et al., 2020). When OS is out of balance due to lack or excess exercise and diet, strategies must be developed to reduce chances of CVD.

Impacts of Reactive Oxidative Species Associated OS

The main ROS believed to be associated with CVD initiation or progression are superoxide ($^{-}O_2$), hydrogen peroxide (H_2O_2), peroxynitrate (ON_3^{-}) and hydroxy radical ($HO^{\cdot}$). Superoxide and H_2O_2 are the two main endogenous ROS that generate free radicals involved in positive metabolic signaling and pathologies when their levels are elevated (Brown & Griendling, 2015). Furthermore, ON_3^{-} can form hydroxy radicals ($HO^{\cdot}$) which is highly reactive and associated with OS and damage to cells. The free radicals that commonly occur are $OH^{\cdot}$, $^{-}O_2$ and nitric monoxide (NO; Holmstrom & Finkel, 2014). Peroxynitrite is a product of NO and $^{-}O_2$ that is associated with cellular damage especially along the cell's membrane causing leakage of calcium into the cell triggering mitochondria and cellular dysfunctions (Beckman et al., 1990). When $^{-}O_2$ is present, NO has greater affinity to react with $^{-}O_2$ than H_2O_2. The lower

concentrations of NO limit potentially necessary vasodilation of endothelia for greater blood flow and increased oxygen delivery. Numerous RNS can form from the intermediates cause cellular membrane damage (Van der Vliet et al., 1996). Formation of ON_3^- support LDL oxidation and nitrotryrosine formation leading to reduced dopamine (DOPA) formation (diurnal effects) and has been implicated as biomarker of atherosclerosis lesion tissues and in plasma (Leonor, 2015). The balance imbalance between OS and antioxidants levels or ratios are considered important in CVD. ROS and RNS serve to protect cells serving as signal transduction molecules and support normal cell activities when there is homeostasis (Janssen-Heininger et al., 2008). If ROS levels become elevated positive and negative consequences occur to phosphatases, kinases (Garcia, 2017), and pathways associated with metabolic growth factors and other mechanisms (Fujino et al., 2007; Sart et al., 2015; Heppner et al., 2016).

Types of Reactive Oxidative Species on Cellular Interactions

A review paper provided an overview of the different types of ROS that are formed as they occur for normal metabolic signaling and when homeostasis is out of balance causing detrimental activities to cells (Cervante Gracia, 2017). The authors suggested that the main causes of CVD forming ROS are mitochondrial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), XO, lypoxidases (LO) and myeloperoxidases (MPO). Furthermore, ROS formation is a cascade of chemical activity leading to CVD where H_2O_2 induces NOX (family of enzymes with different metabolic functions) activation causing xanthine dehydrogenase to form XO and NO_3^- induced $^-O_2$ formation as previously described by Dikalov (2011). Crosstalk or interactions between mitochondria, NOX and NADPH oxidase support greater ROS formation that led to OS and CVD. Especially in cardiac cells, mitochondrial-ROS (mtROS) formed in ETC is a major contributor to ROS production (Assies et al., 2014; Gracia et

al., 2017). The mtROS associated with endothelial dysfunction such as mtROS and NOX-ROS crosstalk where the NOX enzyme (NADPH oxidase) leads to formation of ROS or cause a reverse reaction. The ROS-NOX interaction is associated with endothelia dysfunction as mtROS and $-O_2$ form in unison in endothelia cells.

Under normal conditions, NO is critical for normal vascular function, but NO decreases when $^{-}O_2$ levels increase forming ON_3^- considered a very reactive species causing irreversible cellular damage leading to hypertension (Landmesser et al., 2003). The crosstalk signaling of the chemicals attempt to maintain balance but can also cause ROS to result in cellular damage Gracia et al. (2017). For example, ROS production in ETC that occur in complex I (CI) and complex III (CIII) resulting in pore openings of outer and inner membranes (Mikhed et al., 2015) with CIII-ROS supporting protection of cardiovascular system and CI-ROS production responsible for damage and subsequent CVD (Madungwe et al., 2016).

Mitochondria ROS formation in complexes I and III, are primary sources of free radicals, has been shown that pharmacological (supplementation) preconditioning will upregulate ROS for myocardium protection again ischemic/reperfusion injury (Halestrap et el., 2007; Zhou et al. 2015) but ROS inhibition in postconditioning studies reduced cardio reperfusion injury (Sun et al., 2005; Kin et al., 2004). This indicates that supplementation and type of supplementation must be timed correctly to support cardio injury recovery (Madungwe et al., 2016). Madungwe et al. (2016) reported that hearts treated with glutamate/malate had reduced cardiac recovery and function plus increased myocardial infarction size compared to succinate/ubiquinone (glycerol 3-phosphate) when used. Authors believed that complex I formed cardio-deleterious ROS and cardio-protection in complex III. Thus, following ischemia/reperfusion injury, ROS that formed

in complex III inhibited mitochondrial permeability transition pore opening preventing cellular death.

NADPH Oxidase Associated OS

With the importance of NOX in OS process, a summary of the enzyme complex is reviewed here due to their potential to be identified in metabolomic investigations. The NOX family complexes are involved in many metabolic roles and are major contributors to ROS (Heppner et al., 2016). Their functions include physiological signaling for reducing inflammation, antimicrobial protection, cellular differentiation, and inflammation associated with cellular damage (Amanso & Griendling, 2102). The complexes are formed in membranes of vascular tissue with NOX1, NOX2, NOX4, and NOX5 responsible for ROS formation and NOX2 and NOX4 forming ROS in fibroblasts and cardiomyocytes. Excluding NOX2 the others are formed in vascular smooth muscles of heart tissue (Gracia et al., 2017). NOX4 is involved in stem cells, fibroblasts and cardiac cell migration and growth (Li et al., 2006). All NOX enzyme complexes form $^{-}O_2$ with exception of NOX4 which is primarily associated with H_2O_2 formation. H_2O_2 formed from NOX was found to disrupts tyrosine phosphorylation signaling by thiol oxidation interfere with DOPA formation (Schroder et al., 2012). Reduction of DOPA can alter metabolic process by disrupting nervous system causing dopaminergic neuronal loss and thus cause Parkinson disease and other altered neurological functions. During early stages of cardiovascular dysfunction and atherosclerosis NOX2, NOX4 and NOX5 have greater expression but level off as disease progresses (Guzik et al., 2000; Guzik et al., 2008).

Lipoxygenases and Cyclooxygenases Associated OS

Other ROS contributors to CVD include six lipoxygenases (LOX) that are directly associated with atherosclerosis and ischemic reperfusion (Martel-Pelletier et al., 2003). Two main families of arachidonic acid derivatives as prostaglandins including cyclooxygenase (COX) consisting of isoforms COX 1 and COX 2 and second main family are leukotrienes that are synthesized from 5-lipoxygenase (5-LOX). Both families have major roles as arachidonic acid lipid mediators which exhibit or regulate inflammatory properties. COX converts the arachidonic acid to prostaglandin H_2 (PGH_2) into prostacyclins, thromboxanes, and prostaglandins (Martel-Pelletier et al., 2003). Of those six, 5-LOX and 12/15-LOX activity between select metabolites were reported to support CVD development (Gracia et al., 2017). The family of LOX transforms fatty acids in leukotrienes. Specifically, arachidonic acid provides the substrate for increased CVD supporting formation of hydroperoxides that can be reduced to hydroxides and leukotrienes. Based on carbon structure in arachidonic acid LOX causes different metabolites to form during reactions (Van Leyen, 2013). In vascular cells arachidonic acid is responsible for generating ROS when catalyzed by 5-LOX then 5-HETE and LTA4 leukotriene are responsible for lipid mediation. If 5-LOX is inhibited results in improved ischemia and myocardial infarction but did not inhibit LTs-LTB4 pro-inflammatory molecule that affects critical cell types such as foam cells, T-cells, and endothelial cells (Adamek et al., 2007; Shekher & Singh, 1997). However, in this case 5-LOX causes a negative cellular response due to inflammation by LTA4 (Poeckel & Funk, 2010). Lipoxygenases 12/15-LOX responsible for inflammation and oxidative stress when overexpressed can produce high levels of pro-inflammatory cytokines, IL-6, and TNF-alpha and cause oxygenation of LDL molecules

(Hansson et al., 2006; Wen et al., 2007). A brief review of 5-LOX and its numerous functions was presented previously (Gracia et al., 2017).

Xanthine Dehydrogenase and Xanthine Oxidase Associated OS

Patients with CVD have increased levels of uric acid with xanthine oxidoreductase being essential enzyme responsible for the formation of uric acid (Gracia et al., 2017). To form uric acid xanthine dehydrogenase (XDH) and xanthine oxidase (XO) reduce hypoxanthine and xanthine to uric acid (Figure 2). Under different OS such as in apoptosis, inflammation or mtROS or NOX-ROS, XDH reacts with NAD^+ will form XO (Nishino et al., 2008). If oxygen of XO accepts electrons formation of $-O_2$ and H_2O_2 occurs. The increased levels of XO under the stress factors above supported through formation of NO_3^- via NO and $^-O_2$ reaction. This higher levels of NO_3^- enhance cell damage (Maxwell & Bruinsma, 2001). Therefore, Xanthine and XO increased levels can serve as markers of CVD such as congestive heart failure.

Myeloperoxidase Impacts on CVD and Coronary Artery Disease

Another enzyme associated with endothelial dysfunction is high levels of myeloperoxidase (MPO) that support CVD and coronary artery disease (Anatoliotakis, et al., 2013). This enzyme is considered the primary metabolite causing inflammation and atherosclerosis leading to CVD. MPO mediates negative ions, H_2O_2 forming hypohalous acids (e.g., $HOCL^-$) and hypothiocyanous acid (HOSCN) serves as a potential bacterial control. However, when MPO is upregulated, cellular damage can occur (Van der Veen et al., 2009). $HOCL^-$ formed from upregulation of MPO can cause negative metabolic processes as $HOCL^-$ can reacts with sulfur and nitrogen atoms residues on cysteine molecules present in glutathione (GSH). The oxidation of cysteine changes active sites stimulating or inhibiting enzyme activity

such as metalloproteases (MMP-7) that initiates atherogenesis by disrupting cellular redox homeostasis (Fu et al., 2001). Oxidation of GSH by $HOCL^-$ forming glutathione sulphonamide serves as a marker for MPO activity and indirectly atherogenesis (Pullar et al., 2001). HOSCN formed from MPO can react with thiol causes tryptophan oxidation reducing specific enzyme activity and supports apoptosis (Lane et al., 2010). MPO is primary oxidase within leucocyte of endothelial cells and are directly associated with their dysfunction causing inflammation. Insulin resistance was reported to increase ROS formation and MPO levels in leucocytes that are all associated with OS leading to CVD (Victor et al., 2016). Thus, MPO or its biomarkers appear to be a good indicator of OS.

Benefits of Nutritional Supplements for Reducing OS

Vitamins and other Natural Compound for Reducing OS

Effects of CVD under stress and poor nutrition contribute to obesity leading to a cascade of other diseases such as diabetes contributing to CDV. Proper nutrition that reduces OS supports beneficial metabolic responses increasing improved physical performance for our bodies. Proper dietary training and supplementations that reduce OS include foods such as fiber containing nuts, grains, fish, fruit, and vegetables. In a study of participants with diabetes and CDV, mixed nuts (walnuts, almonds, and hazelnuts) supplementation of 30 g was given daily over 12 weeks. Results showed no significant differences between the consumption group versus control group for antioxidant capacity, oxLDL, and endothelial function (Lopez-Uriarte et al., 2009). However, there was lower lipid peroxidation levels and DNA damage significantly decreased in the consumption group supporting benefits of nuts for reducing metabolic syndrome leading to CVD and potentially other diseases.

There are many studies that evaluated health benefits of phytochemicals from fruits and vegetables for their antioxidant potential (Du et al., 2016; Ghayur & Gilani, 2005; Hooper et al., 2012; Noratto et al., 2015; Pereira et al., 2015; Toh et al., 2013). These studies reported that fruits contain the polyphenol compounds anthocyanins, catechins and tannins. The vegetable flavonoids such as lycopene, spices containing piperine and safranal and the polyphenols catechins, epic-gallocatechin provide protective action and lower CVD. Also, within the fruits, nuts, and vitamins in vegetables such as A, C, D, E, selenium, zinc, and magnesium were reported to provide antioxidant protection reducing oxidative damage and supporting blood glucose homeostasis (Hosseini et al., 2017). It is important to note that fruits and vegetables provide beneficial effects, but some can be detrimental especially if there are toxic levels associated with a given vitamins (Ji et al., 2016). Proper diets having fiber rich foods, vegetables, fruit, whole grains, fish, nuts, red wine can decrease impact of LDLs and need for these dietary exogenous supplementation (Abete et al., 2010).

Supplementations with select vitamins and nutrients coupled with proper diet strategies (e.g., CRD) can provide beneficial health effects resulting in lower hypertension, maintaining blood glucose homeostasis, reduce atherosclerosis forming lipids and many other negative impacts on the body (Waldman et al., 2020). For professionals with high stress related jobs such as police, FF, military personnel, and more recently doctors and nurses since Covid-19 pandemic of 2020. Realistic supportive strategies such as chronic ingestion of select supplementations could improve their hormeric oxidant/antioxidant balances and reduce chronic OS levels maintaining their cardiovascular health. Below the discussion is limited to select supplements such as curcumin, ketones and dietary strategy using CRD protocol and exercise to support health benefits for first time responders such as FF.

Curcumin Supplementation for Reducing OS

Curcumin, a naturally occurring spice found in rhizomes of turmeric (*Curcumin longa*) plants is a polyphenol with a chemical composition of bis-α, β-unsaturated β-diketone, named (E, E)-1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5 dione (Milobedeska et al., 1910; Hatcher et al., 2008). It is known to be a nutritionally safe medicinal pharmacological supplement containing antioxidant, anti-inflammatory, antiarthritic, antiviral, and anticancer properties (Srimal et al., 1973; Sugiyama et al., 1996). Specifically, curcumin can regulate various molecular targets including transcription and growth factors, inflammatory cytokines, protein kinases and other select enzymes (Zhou et al., 2011).

Past studies have questioned curcumin efficacy due to poor bioavailability from low absorption potential and rapid metabolism (Anand et al., 2007). Curcumin molecules will attach to enteral mucosa limit enterocyte uptake, when uptake occurs it is biotransformed before transported into cells or metabolized in the hepatocytes and excreted as bile (Anand et al., 2007). Over the last 10 years the efficacy of curcumin has been improved by using more efficient carriers allowing for transport into the cells (Sathuvan et al., 2017).

With concern of curcumin's bioavailability, studies using carriers were conducted to determine the supplements increase in metabolic efficacy. Essential Turmeric Oil Coated Curcumin (ETO Curcumin) was reported to have 7 to 10-fold higher bioavailability, retained longer in blood than standard curcumin (Toden & Goel, 2017). Improved encapsulation systems or carriers to increase curcumin bioavailability by reducing uptake by organs (Song et al., 2011; Sathuvan et al., 2017) will support greater pharmacological efficacy. Any further discussion related to curcumin efficacy more recent studies reported adequate absorption in gastrointestinal tracts (Hewlings & Kalman, 2017; Singletary, 2020).

Curcumin and ROS Homeostasis

Curcumin supporting ROS homeostasis is an important role of this compound. Details of specific metabolic reactions occurs from three main processes. Hydroxyl radical forms when $OH^{\cdot}$ (hydroxy radical) is formed through Haber-Weiss reaction as $O_2^{-\cdot} + H_2O_2$ react forming OH + OH^-. Another source of $OH^{\cdot}$ is from Fenton reaction where reduces ferric iron to ferrous iron as $Fe2^+ + H_2O_2$ forming $Fe_3^+ + OH + OH^-$ (Wang et al., 1997; Heger et al., 2013). The third way and most important is the ETC at complexes I and III where majority of ROS are formed. Studies that focused on the Fenton reaction reported that curcumin exhibited good and bad results (Miller & Aust, 1989). High doses of curcumin supported greater antioxidant activity, but when using lower rates greater ROS levels occurred (Kunchandy & Rao, 1990). Researchers suggested that the changes occur somewhere between 0.61 and 2.70 µM suppressing formation of $OH^{\cdot}$. Depending upon the dosages of curcumin, like ascorbic acid, can reduce metals such as Fe^{3+} and that curcumin was better in reducing Fe^{3+} when concentrations with ascorbic acid were uniform (Keddy & Lokesh, 1994). When curcumin is structurally changes or is degraded oxidation of metals ceases (Heger et al., 2013).

Curcumin Modulation of Signaling Pathways

The signaling pathways modulated by curcumin contain many molecules and pathways as they directly bind to specific molecular targets or indirectly by affecting their functions such as antioxidant and anti-inflammatory properties. It has been reported that researchers have identified over 30 different proteins curcumin directly interacts involving the regulation of different molecular targets such as lipoxygenase (LOX) (Skrzpczak-Jankun et al., 2003), DNA polymerase (Takeuchi et al., 2006), tubulin (Gupta et al., 2006), and divalent metal ions such as Fe, Cu, Mn, and Zn (Baum & Ng, 2004; Ishihara & Sakagami, 2005). Curcumin molecular

targets and signaling of pathways include transcriptional and growth factors, inflammatory cytokines, protein kinases and other enzymes (Xue et al., 2012). For transcription factors such as nuclear factor kappa B (NF-κB) is a signaling molecule supporting inflammatory responses. Curcumin inhibits NF-κB signaling acting as an inhibitor resulting in anti-inflammatory and anti-cancer responses (Olivera et al., 2012). It has been reported that peroxisome proliferator-activated receptor-y (PPAR- y) involved in energy metabolism regulation is an anti-inflammatory product that increased activation following curcumin ingestion (Voutsadakis, 2007).

To further discuss the potential metabolic versatility as a cancer treatment, curcumin was reported to support reduced cancer cell developed based on many indirect factors associated with tumor development. Growth factors and their receptors affect growth for normal cell differentiation and development (Heger et al., 2013). If unregulated, factor-α (TGF-α) a protein growth factor serves as a ligand supporting unchecked malignant growth. Curcumin inhibited the growth factor expression in cancer reducing cellular differentiation and its antiangiogenic effects critical to cell tissue survival (Zhou et al., 2011). Another growth factors that can be impacted by curcumin is mTOR, a key regulator of numerous cellular processes including protein synthesis and its function for monitoring amino acid/nutrient levels (Tokunaga et al., 2004). Furthermore, curcumin inhibits IGF-1 which stimulates mammalian target of rapamycin complex 1 (mTORC-1) by dissociating scaffold protein raptor from mTOR disrupting coupling between mTOR with reactive substrates (Beevers et al., 2009). Curcumins metabolic effect on mTOR causing suppression of many negative cellular growth responses as related to malignant growth.

Inflammatory cytokines are formed following injury and during the infection processes causing pathological and physiological tissue and organ damage (Mumford & Pugin, 2001). Cytokines mostly associated with inflammation are TNF-α, IL-1β and IL-6 where TNF-α functions as an important in immune cell regulation systematic associated with inflammation (Lockley et al., 2001). Curcumin modulates inflammatory cytokines by regulating transcriptional factors such as NF-κB preventing gene transcription (Chen et al., 2008). Additional studies of curcumin effects on inflammatory cytokines have shown similar suppressive effects that can contribute to reduced inflammation leading to OS (Zhou et al., 2011).

Inflammation and cancer associated enzymes such as 5-LOX, COX-2, and AMP-activated protein kinase (AMPK) are found to be affected using a curcumin diet or as an oral supplementation. AMPK is key protein associated with energy homeostasis, cellular hypertrophy, and down regulation of COX-2 (Chun et al., 2003: Kunnumakkara et al., 2007). COX-2 is induced by inflammatory stimuli supporting synthesis of proinflammatory prostaglandins and cancerous tissues (Seibert & Masferrer, 1994). Curcumin was reported to significantly increase AMPK phosphylation and acetyl-CoA carboxylase associated with formation of malonyl-CoA involved in fatty acid biosynthesis (Kim et al., 2009). Curcumin having hypolipidemic properties through AMPK activation suppresses fatty acid and cholesterol biosynthesis including fatty acid synthase and HMG-CoA reductase that can lead to reduce obesity and lipogenesis, maintaining lipid homeostasis maintaining balanced OS (Liu et al., 2017). With high fat diets curcumin can also stimulate the canonical Wnt pathway inhibiting differentiation of adipose cells but in mature cells attenuated Wnt activity using a lipid enhanced diet (Shao et al., 2012). Specifically, the study reported that a high fat diet with curcumin reduced lipogenesis in liver and inflammation in adipose tissues, while supporting glucose

removal and insulin sensitivity. Based on previous studies, curcumin has the potential to enhance or inhibit many different enzymes to either activate or prevent different metabolic functions generally all resulting in health benefits reducing OS and CVD. However, in the study by McAllister et al. (2018) using exogenous curcumin to reduce transient OS in FF did not show any difference concentrations of several antioxidant associated metabolites. However, in a later investigation curcumin supplementation using military personnel there was significantly reduced biomarker levels associated with OS (McAllister et al., 2020).

Curcumin Supplementation to Reduce Exercise Induced Transient OS

To evaluate the benefits of exercise and nutrient supplementation for reducing CVD associated factors, military personnel participated in a dual stress challenge test evaluating mental and cycling stress testing plus curcumin supplementation (McAllister et al., 2020). In this study, OS markers such as SOD, H_2O_2, and advanced oxidation protein products (AOPP) 30 and 60 minutes after exercise were significantly lower. Basham et al. (2019) reported that following eccentric muscle exercise protocol test and oral curcumin supplementation determined that nutrient blunted creatine kinase, and muscle soreness ratings were lower suggesting lower muscle damage (Basham et al., 2019). In the investigation, antioxidant capacity, malondialdehyde (MDA), and TNF-α levels were similar between the curcumin supplementation and placebo groups. With the variable results between these two studies may suggest that rates of exogenous curcumin affect efficacy and its ability to reduce OS. To increase the metabolic information from the curcumin supplementation study, ^{1}H NMR study evaluating the stored FF blood may provide additional metabolic data impacting the transient OS following an exercise protocol reported by McAllister et al. (2018).

Ketone Body Production and Associated Metabolites

Under prolonged fasting, starvation or evaluating specialized diet such as CRD, triglycerides from adipose tissue are reduced releasing free fatty acids (FFA) from triglycerides are transported to the liver mitochondria where ketone bodies form (Veech 2014). Ketone bodies include β-hydroxybutyrate (ketone ester), acetoacetate, and acetone. Reduced insulin levels prevent glucose availability to cells triggering β-hydroxybutyrate synthesized in the liver that are transported to non-hepatic tissue forming acetyl-CoA that contributes to citric acid cycle and energy production. Ketogenic amino acids lysine and leucine can be degraded directly to acetyl-CoA and are readily identified in blood using metabolomic platforms such as ^{1}H NMR (Yudkoff et al., 2014). Understanding the amino acids associated with insulin sensitivity or downregulation are critical when evaluating metabolomic biomarkers that may impact OS (Noguchi et al., 2010). Strick ketogenic amino acids are phenylalanine, tyrosine, tryptophan, isoleucine that can contribute to acetyl-CoA formation. Under glucose homeostasis increased levels of the glucogenic amino acids would likely occur for arginine, glutamate, glutamine, histidine, proline, valine, methionine, aspartate, asparagine, alanine, serine, cysteine, and glycine (Thomas et al., 1982).

Levels of pre-ketosis to full ketosis occurs when the body is in a starvation state or with prolonged exercise resulting in energy stress and from ketone supplementation or their combinations (Stubbs et al., 2017). Following initiation of ketogenesis, acetoacetate levels are highest in blood reacting with acetoacetate decarboxylation and quickly converted to β-hydroxybutyrate becoming more abundant as ketosis proceeds leveling off with more balance between the ketone bodies (Stubbs et al., 2017). With reduced CRD or ketogenic diets, a cascade of metabolic processes are initiated including mTORC1 inhibition and preventing

regulator peroxisome proliferator activation of the receptor α (PPARα) a transcriptional factor involved in hepatic ketogenesis (Grabacka et al., 2016). PPARα, γ regulates cell responses based on nutritional states (fatty acid levels) and are transcription factors preventing encoding for amino acids, lipid, and carbohydrate metabolism when mTORC1 is inhibited for fatty acid and protein synthesis. Carbohydrate restricted diets will not cause the same heightened responses as ketosis, but metabolic changes will occur with fluctuating levels of ketone bodies and amino acids shifting as energy levels are reduced with exercise and from the dietary challenge.

Ketone Esters and Salt Supplementations

Supplementation using ketone salts and esters or when under dietary nutritional limitations such as CRD are believed to have beneficial health consequences. The salt and ester supplements have been reported to enhance weight loss by reduction of adipose tissue densities from utilization of triglycerides broken down into fatty acids for energy production. During normal ketogenesis, it was noted that ketones can increases power of ΔG of ATP indicating an indirect need for fatty acid utilization (Veech, 2014). Ketosis resulted in increased energy hydrolysis (Cahill & Veech, 2003) and lowers acute insulin resistance at least in injured or infected tissues (Li & Messina, 2009), and supporting ROS homeostasis (antioxidants: ROS). Furthermore, reduced triglyceride levels that are components of LDL (cholesterol), support greater HDL densities contributing to reduced obesity (Paoli et al., 2013; Gershuni et al., 2018). Ketosis naturally reduces insulin through low blood glucose levels causing reduced transcription of genes responsible for enzymes such as HMG-CoA reductase necessary for cholesterol synthesis (reduced LDL) and preventing or reducing transcription of the responsible carbohydrate element binding protein from entering the nucleus of liver cells causing enzyme activation for fatty acid synthesis (Nakagawa et al., 2013).

β-hydroxybutyrate is the main source for supplementation as the ketone of choice since it lessens the potential from acidosis and is very stable compared to acetoacetate which becomes quickly decarboxylated (Fritzsche et al., 2001). β-hydroxybutyrate supplementation reducing OS, improve insulin sensitivity, inflammation and subsequently CVD (Soteriades et al., 2011; Shimazu et al., 2013). Using exogenous supplementation of β-hydroxybutyrate acts as a histone deacetylase inhibitor increasing acetylation of promoter regions on genes responsible for transcription of antioxidants such as superoxide dismutase, catalase, and metallothionein supporting homeostasis with ROS (Shimazu et al., 2013). Furthermore, ketone supplementation or ketosis through diet cause inhibition of transcriptional receptors that initiate NAD-linked histone deacetylase Sirt 1 to be inhibited causing hyperacetylation of tumor suppressor gene, p53 an activator for an apoptotic pathway supporting tumor suppression (Zhao et al., 2008; Veech, 2014).

Exogenous ketones supplements are primarily in the form of ketone salts and ketone esters (Clark et al., 2012; Harvey et al., 2019). It is still uncertain if exogenous supplementation is better that dieting alone or if this type of ketone supplement supporting ketosis. The main concern with salt formulation is possibility of alkalinizing metabolic accumulation effect on blood pH and renal function. Ketone generally have a sodium (Na^+) attached while a ketone ester has double bonds attached to oxygen. Ketone esters are β-hydroxybutyrate and acetoacetate but the two can potentially cause different metabolic responses used for different purposes and further studies are necessary to evaluate both oral ketone supplements.

When ketone bodies exceed metabolic capacity in blood then ketoacidosis occurs with negative metabolic health consequences. As ketone bodies levels exceed hormeric balance, high cation levels from uric acid formation occurs causing gout (Sampath et al., 2007). If free fatty

acid concentrations in blood increase, ketone body formation become greater (Cahill & Veech, 2003). In diabetic conditions, free fatty acids often exceed the capacity for them to be metabolized, common during insulin deficiency with type 1 diabetes or late-stage diabetes type 2, resulting in ketoacidosis which is life-threatening (Ward, 2015). Without sufficient insulin glucose released from liver accumulates in the bloodstream and the body requires other energy sources. Oxaloacetate which normally is formed from pyruvate during glycolysis becomes low without sufficient glucose for glycolysis to occur and oxaloacetate is preferentially use in gluconeogenesis making it unavailable to the citric acid cycle. Free fatty acids are next available source for energy by forming acetyl-CoA or can begin to form ketone bodies in excess since oxaloacetate was unavailable. Another disease associated with ketone bodies occur when ketogenic diets improperly administrated or monitored can result in hypercalciuria causing bone demineralization and excretion of calcium from kidneys and acidosis causing kidney stone formation from the excess calcium in the body (Hawkes & Levine, 2014).

Ketone Supplementation Impacts on Exercise Induced Transient OS

Ketone diets have been shown to improve mitochondrial functions from selective uncoupling of proteins preventing OS formation (Hyatt et al., 2016). In studies evaluating ketone supplementation of exogenous nutrient sources showed ergogenic benefits in exercise performance in many cases, but results varied depending upon study protocol and exercise testing methods for performance (Cox et al., 2016). A review paper covered 16 studies (112 participants; 109 males and 3 females) examined the impact of ingesting ketone compared to controls and exercise performance (lower-body and endurance power; Margolis & O'Fallon, 2020). Overall, it was determined that there were significant differences between exercise performances, supplementation with ketone esters (β-hydroxybutyrate) but with lesser benefit

from ketone salts compared to controls. Across the 16 studies, results varied (3 positive, 10 nulls, 3 negative) making it difficult to conclude true benefits for exercise performances and supplementation suggesting that recommendations for using ketone supplementation for improve physical performance currently was inappropriate. Research using ketone supplementations reported increased plasma β-hydroxybutyrate concentrations but did not improve cognitive or physical performances (Evans et al., 2019), and increased D- β-hydroxybutyrate supplementation did not benefit endurance athletes (Shaw et al., 2018). Review paper by Margolis & O'Fallon (2020) suggested there were metabolic health benefits associated with using the ketone supplements, but overall results varied across studies. The authors suggested that variations in ketones, dosage and application timing and exercise regime contributed to inconsistent results. In the review paper by Margolis & O'Fallon (2020), Waldman et al. (2018) was listed within the three positive studies using ketone monoester supplementation that improved performance, but that ketone salt evaluated had no positive effects. Not included in the review paper was a 7-day ketone salt ingestion study evaluating changes in targeted antioxidant and oxidant levels following exercise protocol supporting transient OS (McAllister et al., 2019). The research evaluated cardiorespiratory responses and exercise stress responses with FFs in personal protection equipment. Results indicated that ketone salts did not reduce OS based on the 7-day supplementation, but HR was lower. It was thought that ketones decrease sympathetic tone causing reduce HR supporting cardiovascular function (Kimura et al., 2011). It was also noted that as OS levels from exercise decreased glutathione levels immediately post-exercise and at 30-min after but returned to normal at 24 hours. Dearlove et al. (2019) compared the effect of ketone ester consumption just prior to incremental bike ergometer to exhaustion and did not reduce performance due to slightly elevated ventilation and early hyperventilation during

exercise. Ketone supplementation blunted stagnation and performance from overreaching symptoms in a study using high intensity interval training (HIIT) and intermittent endurance training on cycle ergometer (Poffe et al. 2019). Cox et al. (2016) evaluated ketone supplementation versus a long-chain FAT diets with exercise found that ^{1}H-NMR untargeted metabolite data supported improved athletic exercise performance. Metabolite composition in that study indicated that acute nutritional ketone-initiated ketosis provided some athletes with increased physical performance but impacts on OS were not discussed.

Restricted Carbohydrate Diet

The CRD is a ketogenic diet plan that is designed to decreases amount of glucose for energy production and encourages metabolic degradation of stored triglycerides, glycerol and fatty acids, and low levels of protein that eventually lead to formation of ketones bodies. The dietary strategy enhances metabolic and cardiovascular systems supporting hormeric balance between antioxidants and ROS levels (Meydani et al., 2011; Paoli et al., 2013; Greco et al., 2016). Causing decrease glucose and insulin levels from the CRD and suppressing lipogenesis activity associated with fat accumulation. During ketosis, insulin/insulin-like growth factor-1(IGF-1) pathway associated with mitochondrial dysfunction is inhibited resulting in improved mitochondrial function and subsequent lower ROS levels (Shimazu et al., 2013; Greco et al., 2016). The IGF-1 factor is associated with many negative metabolic processes and even implicated in cancer cell mitogenesis, acne formation, inflammation, and type 2 diabetes that is suppressed with CRD. The diet has even been used for treating epilepsy with various degrees of success (Paoli et al., 2013). Using CRD with aerobic exercise, insulin sensitivity improved by 20% and IGF-1 levels decreased by 9% supporting lower metabolic syndrome levels and CVD (Nishida et al., 2010).

Depending upon the type and duration of exercise affects what energy sources body will utilize. For endurance training using traditional dieting, carbohydrates are main sources that are oxidized by active skeletal muscles (Cermak & Loon, 2013). Exercise performance studies have showed variable results using CRD, with indirect benefits noted for OS and CVD (Waldman et al., 2020). In that study, cardiometabolic profiles such as triglycerides (TAG), malondialdehyde (MDA) and AOPP decreased and total cholesterol associated with HDL increased. These biomarkers indicated a possible reduction in OS stress leading to reduced CVD while no specific mention on athletic performance from the study was noted. Similar results from 5-km running plus CRD diet challenge had no diet-related exercise performance differences (Prins et al., 2019). In another study, improved body composition and fat oxidation were observed using CRD with a trend for improved runner performance in a 5-km course (Heatherly et al., 2018). Contrary to the above research on CRD, exercise economy performance of elite race walkers was impaired (Burke et al., 2017). An endurance athlete who followed a CRD for two years was tested using carbohydrate ingestion during a phased high-intensity training session for 7-weeks with results based on timing of the activity (Webster et al., 2018). No improvement was identified from 30-sec sprint power runs considered short-term. At 4 to 30 min, sprint power improved with a reduction time in 100-km trial. Chang et al. (2017) suggested that a low carbohydrate high fat diet (LCHF) long-term caused metabolic changes (adaptations) enhancing performance and health due to weight loss. Many of these studies suggest the importance of metabolism and metabolic changes associated with exercise and nutritional considerations. Increasingly metabolomic data in such studies using the ^{1}H NMR platform would provide important supportive information for assessing not only exercise performance but also the impacts of the physical activities on OS and cardiovascular health.

Anthropometric Metabolic Data

Exercise performance research has been conducted using anthropometric data such as body mass and height x weight ratio to determine BMI. Cardiorespiratory data can be obtained using a 5-stage cycling graded exercise determined substrate oxidative rates followed by Wingate sprint to determine anaerobic power output for CO_2 production. From these exercises the respiratory exchange ratio (RER)= VCO_2/VO_2 can be determined measuring carbon dioxide exhaled to amount of oxygen consumed. The ratio is a fitness indicator by providing amounts of fat to carbohydrate consumed during levels of exercise intensity or stress related situations and for evaluating impacts of different nutritional regimes. The RER values provide information of a person's fitness (trained or untrained) or adaptability to different levels of stress (Ramos-Jimenez et al., 2008).

Exercise physiology literature contains scores of nutritional studies lists where anthropometric and traditional performance measures were collected to understand impacts of nutrient or diet supplements on untrained and trained individuals. These data did not provide information that could be used to determine treatment effects for measuring OS. Glycemic load was used to quantify dietary data of select food servings was reported to be a marker for OS (Limkunakul et al., 2014). The glycemic load showed a strong correlation with blood plasma MDA and F2-isoprostanes as OS biomarkers (Hu et al., 2006). With improved laboratory methods blood markers were used to evaluate dietary supplementation and effects of caloric restriction on performance and health (Meydani et al., 2011; Greco et al. 2016). More recently, metabolic biomarkers can assess changes in OS in three FF studies including glutathione (GSH), oxidized glutathione (GSSG) catalase (CAT), superoxide dismutase (SOD), AOPP; McAllister et al., 2018; McAllister et al., 2019; Waldman et al., 2020) and malondialdehyde (MDA) for

measuring lipid peroxidation (McAllister et al., 2019). These methods to identify and quantify the targeted OS related metabolites but did not always provide definitive information. The scope of the data was limited and advanced bioanalytical methods such as genomic and proteomics could provide additional metabolite diversities and concentrations (Nicholson et al., 1999). The authors believed that broader scope of metabolites would provide holistic approach using untargeted metabolome for understanding dynamic cellular functions of whole organism including OS biomarkers. With availability of other technology such as nuclear magnetic resonance (NMR) and mass spectrometry, liquid chromatography-mass spectroscopy (LC-MS) and gas chromatography-mass spectroscopy (GC-MS) metabolomic analysis of entire metabolites compositions within human blood can now be conducted.

Metabolomic Studies Using ^{1}H NMR

Metabolomics Defined

The terms metabonomic and metabolomic by some scientists are considered the same but there are distinctions between the two. Metabonomics is defined as measurement of metabolome temporal changes associated with specific intervention from smaller numbers of metabolites. Metabolomics is a concurrent assessment of metabolites that are at endpoints of upstream metabolic pathways of phenotypic responses (Sakaguchi et al., 2019). These small molecule metabolites (< 1.5kDa) forming products of enzyme (coenzyme) mediated reactions consist of substrates, intermediates, signaling agents, and reactants (Dunn et al., 2011). Metabolomics attempts to measure the whole metabolome to get overall coverage of available data from specific platforms such as ^{1}H-NMR. Metabolome as defined is a comprehensive study of identification and quantification metabolites within a biological system (Fiehn, 2002; Shah et al., 2015). Metabolomics is a member with a group of omi including genomics, transcriptomics, and

proteomic. Metabolites as biomarkers are a form of phenotypic expression present in organism (human) biological samples that can be used to define biochemical mechanisms as affected by environment, mental and physical stress, and nutritional regimes.

Introduction to ^{1}H-NMR Spectroscopy

This technology provides physical observations which uses isotope hydrogen-nuclei from the molecules of organic substances by utilizing the magnetic properties of their nuclei. Nuclei consistent of magnetic field that are exposed to a weaker oscillating magnetic field inducing an electromagnetic signal. The nuclei that are positively charged spin on their axis creating a tiny magnetic field with H and C. The fields are aligned either with the external field or oppose the external magnetic field. Hydrogen atoms act as magnets that are brought into resonance. To define a specific metabolite the CH_3 occur in different external magnetic field than hydrogens attached to oxygen such as OH causing different peaks associated with the hydrogen atoms it is attached. A standard called tetramethylsilane (TMS) is used to establish a reference peak and is always at zero. It contains four CH_3 that are attached to the same centralized silicone (Si) atom. TMS has 12 hydrogen atoms that are equivalently joined to the same molecule forming a single but strong sharp resonance peak due to its high number of H atoms, located to far right of each NMR graph. Electrons for C-H bonds require an increase in the magnetic field bringing them into resonance that are called chemical shifts. The chemical shifts are measured in parts per million (ppm). A peak with chemical shift of 3 indicates that H atoms responsible for the peak will need a magnetic field 2 millionths lower (downfield) in the field needed for TMS to produce resonance. The peak information can be processed using known databases or in programs that can refer to human blood samples.

^{1}H NMR For Human Blood Metabolic Analyses

In the case of human nutrition, health, and exercise response (stress) studies ^{1}H NMR metabolomic data (targeted and untargeted) has been collected for whole blood (Gowda et al., 2018), urine (Holmes et al., 1994; Nicholson et al., 1999), saliva (Lindon et al., 2000), and pathological tissue (Lindon et al., 2000). Specifically, NMR studies are now possible for understanding human health status using whole blood, plasma, or serum to obtain metabolite fingerprints of OS, inflammation, diabetes, cancer, OS, or other disorders. Initially, preliminary research using NMR was conducted to first optimize the methodology and accuracy of NMR from the more expensive and labor-intensive mass spectrometry (MS) platforms (Gowda et al. 2018). It was determined that for untargeted studies, metabolites were underrepresented due to their binding to plasma and serum proteins (Nicholson & Gartland, 1989; Gowda & Rafferty, 2014; Gowda et al., 2015). Nicholson & Gartland (1989) showed that histidine, phenylalanine, and tyrosine would bind to macromolecules of bovine and human blood plasma and serum with the aromatic amino acids tyrosine and phenylalanine not detected at neutral pH level but would provide weak signals at pH 7.6. Glutamine an important indicator of ROS levels is cycled to pyroglutamic acid during ultracentrifugation of blood serum affected concentration accuracy of this metabolite with ^{1}H NMR platform (Gowda et al., 2015). Using ultrafiltration of serum showed a 10-74% decrease in concentrations of tryptophan, benzoate, and 2-oxoisocarproate compared to methanol protein precipitated serum (Gowda and Raftery, 2014). In later studies using whole blood compared to plasma, additional metabolites were detected such as the coenzymes NAD^{+}, NADH, $NADP^{+}$ and NADPH, energy coenzymes adenosine triphosphate (ATP), ADP (adenosine diphosphate) and adenosine monophosphate (AMP); and antioxidants GSH (glutathione) and GSSG (oxidized glutathione; Gowda & Raftery, 2017; Gowda, 2018).

These same coenzymes could not be detected in plasma and were often lost prior to NMR analysis due to the metabolites inherently low concentrations and poor stability. Complicating data collection accuracy even further, coenzymes NAD^+ and NADH diminished ~ 50% within 24 h as shown from time dependent studies (Gowda et al., 2016). In addition, concentrations of the coenzymes from plasma generally occurring below 6.3 ppm causing overlap with other metabolite spectra making it impossible to differentiate the coenzymes from those metabolites that are in greater abundance in whole blood. To overcome the concern for evaluating NAD^+ and NADH a special protocol is recommended for the important coenzymes but whole blood is required immediately following sampling (Gowda et al., 2016). The need to optimize NMR protocol for blood samples using different methods to identify the metabolites has improved accuracy of targeted data but optimization for specific metabolite defeats the purpose of metabolome fingerprinting. Data acquisition must then be defined as to need of specific targeted groups of metabolites versus untargeted prior to when blood samples are collected.

Sports Related Metabolomic Data

Metabolome fingerprints can also be used temporally to increase the databases for various platforms such as ^{1}H-NMR and GC-MS to enable comprehensive searches of importance targeted or untargeted metabolites/concentrations. Both methods are very effective for different exercise fingerprinting research, but NMR sample preparation requires minimal processing for biological fluid and tissues. Whereas GC-MS is expensive and requires several different layers of techniques for various purposes and requires different resolution using different platforms (Duft et al., 2017). The possibility of using NMR metabolomics in human health and exercise related fields are becoming increasingly common. Metabolomic fingerprinting was first conducted over 40 years ago using GC-MS in human blood and urine samples (Dunn et al.,

2011). However, exercise-based metabolomics investigations of human athletes were first published using GC-MS platform in 2010 (Lehmann et al., 2010; Lewis et al., 2010). Lewis et al. (2010) measured >200 metabolites within several pathways pre- and post-exercise with plasma indicators belonging to glycogenolysis, citric acid cycle, lipolysis and niacinamide modulators of insulin. Using moderately intense exercise in humans, transient increases in targeted lipid metabolites from skeletal muscle tissue showed increases in medium chain acylcarnitines of β-oxidation, a dominant biomarker of fat oxidation (Lehmann et al., 2010). More recently, the metabolome of 80 professional football players were evaluated for internal and external load using urinary metabolic profiles (Quintas et al., 2020). Results from study showed significant associations between urine metabolic profile and internal alterations of biochemical pathway and training adaptations. Metabolites associated with external load were steroid hormone intermediates, phenylalanine pathway metabolism intermediates tyrosine involved in dopamine and catecholamines formation, tryptophan for niacin coenzymes and serotonin and melatonin.

Over the last 10 years many metabolomic studies evaluating impacts of different exercise and nutritional supplements on exercise performance, OS, and cardiovascular health were summarized in several comprehensive review papers. Sakaguchi et al. (2019) did an appraisal of 24 high quality research studies over last 10 years on metabolome variations from different exercise protocols. In that review, 63% focused on acute prolonged and intense exercise. Overall, the results showed large changes in diverse lipid metabolites within few hours post-exercise but returned to pre-exercise levels within 24 h across all metabolites. In an NMR study, acute exercise (<60 min) protocol identified modest shifts in metabolites between recreation males and soccer athletes following intense and prolonged exercise (e.g., Zafeiridis et al., 2016).

Immediate post-exercise resulted in an upregulation of carbohydrate-lipid metabolism and citric acid cycle following aerobic long and short interval exercise. Meisser et al. (2017) determined that following prolonged exercise at high altitude resulted in plasma levels of glucose, glutamine, alanine, and branched-chain amino acids (BCAA) that decreased suggesting protein pathway involvement while lipid use was like those at sea level.

The second major review paper comparing different metabolome following exercise was presented by Duft et al. (2017). The review compared 44 published exercise metabolomic studies that were subdivided into four categories including metabolic responses to physical exercise, supplementation and physical exercise, sports performance, and physical exercise and disease. The metabolomic studies review included targeted or untargeted metabolite fingerprinting (Duft et al., 2017). A total of 17 studies employed NMR while the other 27 used GC-MS spectrometry. Using ^{1}H NMR analysis, a HIIE exercise protocol showed that the energy pathways from glycolysis and citric acid cycle had greatest metabolic changes during aerobic long-interval (3 min) exercise protocol (Zafeiridis et al., 2016). Whereas Danaher et al. (2016) used high intensity exercise (HIE) to simulate physiological remodeling reported significant metabolite differences post-exercise for lipids such as cholesterol, and lactate from glycolysis during recovery.

Supplementation and physical exercise are the second subfield defined by Duft et al. (2017) relating to metabolomic studies. Of these investigations, 89% using acute exercise challenges and 78% aerobic exercise. In one metabolomic study, exercise protocol followed by a carbohydrate drink and caffeine found significant differences in plasma metabolites and concentrations pre- and post-exercise (Kirwan et al., 2009). Results using green tea, catechins and caffeine capsules taken 2 h prior to an exercise protocol conducted across 28-days reported

that β-hydroxybutyrate increased immediate after exercise but no significant acute effects occurred on endogenous metabolites (Jacobs et al., 2014). A milk supplementation using whey protein and calcium caseinate ingested before a strength 1 RM protocol resulted in dynamic plasma metabolic changes in levels of alanine, β-hydroxybutyrate, branched chain amino acids (BCAA), creatine, glucose, glutamine, glutamate, histidine, lipids, and tyrosine post-exercise using NMR (Yde et al., 2013). Miccheli et al. (2009) using rowing to dehydration protocol followed by a post-exercise sport drink containing green tea, differences in glucose, citrate, and lactate levels from plasma and from urine acetone, β-hydroxybutyrate and lactate using ^{1}H NMR.

Sport performance is the third category or subfield of exercise metabolomic studies listed by Duft et al. (2017). About 20% of articles used in this subfield were specifically related to sports performance evaluating variations in metabolome following exercise protocols. In one study, significant endogenous metabolite differences occurred between rowers and healthy control subjects including select amino acids (glutamine, phenylalanine, tyrosine), lactate, free fatty acids associated with glucose metabolism, glycolysis and citric acid cycle, amino acid, and lipid metabolites (Yan et al., 2009). Specifically in that study, OS associated metabolites were assessed using PCA and PLS-DA comparing their concentrations of γ-glutamyl cycle intermediates cysteine, glutamic acid, and pyroglutamic acid supporting synthesis of glutathione varied between the rowers and control subjects. In another study, soccer players performance was assessed using a Yo-Yo test saw no differences between good and poor performers and that metabolic changes occurred from saliva following the test (Santone et al., 2014). Using blood plasma, untargeted metabolomic analysis after a 75-km cycling time trial caused a 3.1-fold increase in the lipid metabolites 13-HODE + 9-HODE and cytokines (IL-6, IL-8, IL-10, TNF-α) responsible for transient OS and inflammation, (Neiman et al., 2014). Many studies such as these

show the benefits of using metabolomic data for evaluating sports performance, but additional studies are necessary to optimize the results for specific needs of the designed research.

Exercise related to disease is the fourth subfield category by Duft et al. (2017). Studies in this category were primarily chronic related since they targeted metabolites associated with diabetes, insulin resistance and related obesity. In one study, a glucose intervention improved insulin sensitivity comparing glucose and lipid concentrations in blood as supported using partial least squares orthogonal discriminant analysis (OPLS-DA; Kuhl et al., 2008). Another study evaluated a HIIT protocol using older females showed that urea cycle metabolites, carnitine metabolism, and aromatic amino acids decreased after glucose supplementation (Kuehnbaum et al., 2014). The focus of these studies was not to observe metabolic changes to exercise but for biomarker discrimination associated with the morbidities. Different metabolome statistical methods were used to evaluate the metabolite data from those studies, but some multivariate analyses were presented while other data was mentioned in more generalities as reported by Duft et al. (2017). The use of multivariate analyses has a role in metabolomics thus far as supported by the literature, but actual value of these statistical analyses will depend upon the studies purpose.

Multivariate Analysis

Overall, multivariate analyses are primary statistical approach for comparing metabolite or metabolome changes following different exercise (physical stress) protocols, supplement usage, or their combination. In the review by Duft et al. (2017) several bioinformatic methods were suggested including Principal Component Analysis (PCA; Jolliffe & Cadima, 2016), Partial Least Squares Discriminant Analysis (PLS-DA), Partial Least Squares Orthogonal Discriminant Analysis (OPLS-DA; Sun et al., 2014) and less used Pathway, Enrichment, and Integrated

Pathway Analyses to study metabolic pathways based on fingerprints (Xia et al., 2015). General discussion for each of these statistical methods can be found in the article by Duft et al. (2017). When actual metabolite concentrations comparisons are needed additional statistical methods using various forms of analysis of variance (ANOVA) must be performed to validate significant metabolite changes associated with exercise and supplementation.

^{1}H NMR Metabolomic Untargeted Analyses Using Firefighter Blood Plasma

Recommendations must be developed to help FF improve their cardiovascular health leading to greater physical stability or performance under often very stressful situations. Using exercise and nutritional supplementation support positive metabolic alterations of the human metabolome reducing impacts of obesity, diabetes, OS, and other factors leading to CVD and cancers. Many of which are metabolites that serve as signaling molecules causing beneficial or negative downstream cellular consequences impacting cardiovascular health. To maintain balanced metabolome in daily life requirements for FF, research and education recommendations are necessary for improving the performance and cardiovascular health of first responders by reducing OS overall as a primary goal. Metabolomic data can be readily available using the NMR analytical platform that is a fast and reliable tool to understand the beneficial or negative metabolic impacts from studies using nutrition and exercise. However, it is uncertain if specific metabolites and their concentrations can be correlated supporting reduce OS and further research is necessary to delineate specific NMR protocols necessary to answer different metabolic questions. Metabolic studies using stored blood plasma compared to whole blood or serum is currently more exploratory determining untargeted metabolites than targeted ones for OS levels impacting CVD (Gowda & Rafferty, 2014; 2017; Gowda et al., 2015; Gowda et al., 2016; Gowda et al., 2018). Biomarker metabolites of OS are either identified as being directly

associated exogenous or endogenous antioxidants or indirect intermediates or signaling molecules affecting OS health patterns. Multivariate analyses PLS-DA and OPLS-DA models show group relationships are used to determine the relevant changes in metabolome experimental groups and potential biomarkers within groups. To validate these potential biomarkers of interest, their concentration means are then subjected to analysis of variance (ANOVA) to determine significant differences comparing the study treatments (Sun et al., 2014). This includes physical activity or exercise, supplemental or pharmacological treatments, and dietary adjustments that can be used to provide recommendations to support better health and performance for first responders such as FF.

CHAPTER III

^{1}H NMR ANALYSIS OF FIREFIGHTER BLOOD PLASMA METABOLITE MARKERS FOLLOWING 28-DAY-CARBOHYDRATE RESTRICTED DIET

Abstract

Previously, a 28-day-carbohydrate restricted diet (CRD) challenge using 15 firefighters (FF) was conducted to determine the diets impacts on improving their cardiovascular health and exercise performance. During that study, blood plasma samples were obtained just prior to a familiarization session, pre-CRD and post-28- CRD sessions. The samples compared targeted OS biomarkers resulting in several positive benefits associated with the diet. In the current study, subsamples of the blood plasma stored for 24 months at -80°C were reevaluated using ^{1}H NMR to determine the untargeted metabolites present from the three sessions. A total of 40 metabolites were consistently identified from the plasma samples and 7 others were detected intermittently. Of those consistently identified were amino acids, ketone bodies, branch chain amino acids (BCAA) and other metabolites covering multiple metabolic pathways. Analysis of variance (ANOVA) determined that ketone bodies acetone and β-hydroxybutyrate and the branch chain amino acids (BCAA) isoleucine, leucine, and valine were significantly upregulated post-28-day-CRD. β-hydroxybutyrate, isoleucine, and leucine have been reported to support reduced OS. To visualize the metabolite concentration results, multivariate analyses were conducted using principal components analysis (PCA), partial least squares-discriminant analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). PCA model

was unable to distinguish the 40 metabolites into groups between familiarization, pre-CRD, and post-28-CRD sampling times. Cross-validation for PLS-DA and OPLS-DA using Q^2 coefficient, and permutation test did not support either model for distinguishing the metabolite concentration groupings between sessions indicating limited metabolite concentration variations.

In conclusion, ^{1}H NMR metabolites identified from the stored FF blood plasma did not show direct benefits for reducing OS. Indirect biomarkers such as β-hydroxybutyrate, isoleucine and leucine detected in this current study were previously reported to reduce OS metabolism. However, it is believed that metabolic data obtained during ^{1}H NMR must be coupled with targeted metabolites, such as specific antioxidants, in future studies. Blood plasma samples, shored short and long-term must be further studied to determine the accuracy and usefulness of the results when assessing metabolic impacts on OS and antioxidant defense associated with cardiovascular risk in FF.

Introduction

Firefighters (FF) were reported to have some of the highest levels of obesity and cardiovascular disease (CDV) in the United States (Poston et al., 2011; Dobson et al., 2013). The progression to CVD resulted in line-of-duty morbidity of 45% (Smith, 2011; Soteriades et al., 2011) with the underlying causes including low fitness levels, increased obesity, diabetes, hypertension, and other CVD factors. In addition to poor health of many veteran FF, physiological effects such as oxidative stress (OS) from intense physical activity, psychological stress, temperature extremes, lack of exercise, and poor nutrition can all lead to the high morbidity rates. In total, these stressors can cause inflammation increasing the potential for CVD through downstream metabolic changes that can be detected using targeted and untargeted metabolomic studies.

These negative metabolic states leading to inflammation and atherosclerosis can naturally be modified or prevented depending upon the genetics of individual FF under daily stressors, by employing diet modifications and nutrient supplementation coupled with regular exercise regime. These health modifications can result in positive health changes reducing the damage from the underlying causes (Grundy et al., 2005; Alshehri, 2010; Kaur, 2014). Furthermore, normal exercise is known to increase transient OS from ROS that support cellular signaling necessary for muscular adaptations (Powers et al., 2020), reduced hypertension with increased cellular repair signaling for remodeling of damage cells (Radak et al., 2013). In addition to the stressors, dietary programs and exercise will cause metabolic changes that can be monitored directly or indirectly by detection of intermediates associated with the adjusting metabolism.

Dietary intervention programs, such as the 28-day-carbohydrate restricted diet (CRD) showed significantly improved concentration changes in metabolic markers of CVD (Waldman et al., 2020). This study was one of first that employed controlled dietary interventions with exercise that had FF participation. However, past reports using applied behavioral studies and exercise activities by first responders are numerous (Dobson et al., 2013; Abel et al., 2011; 2015; Pawlak et al., 2015)

The CRD can be labelled as a ketogenic diet plan designed to decreases amount of glucose for energy production and encourages metabolic degradation of stored triglycerides, glycerol from fatty acids, and limited levels of protein. This type of diet can eventually support the formation of ketones bodies even though full ketosis does not always occur (Waldman et al., 2020). This dietary strategy further enhances metabolic and cardiovascular systems supporting homeostasis between antioxidants and ROS levels (Meydani et al., 2011; Paoli et al., 2013; Greco et al., 2016). From whole blood, metabolic intermediates advanced oxidative protein

products (AOPP), malondialdehyde (MDA), and triglycerides were significantly lower and increased total cholesterol and high-density lipoproteins (HDL) but no differences in low-density lipoproteins (LDL) occurred following 28-day-CRD (Waldman et al., 2020). Using new ^{1}H nuclear magnetic resonance (NMR) detection methods, a large number of metabolites from stored blood samples that can be detected to might provide additional details related to benefit of a CRD for improved CVD health. With availability of other technology such as NMR and mass spectrometry including liquid chromatography-mass spectroscopy (LC-MS) and gas chromatography-mass spectroscopy (GC-MS) the entire metabolome can be mined and is known as metabolomics (Nicholson et al., 1999). Metabolomics is a concurrent assessment of metabolites that are at endpoints of upstream metabolic pathways of phenotypic responses (Sakaguchi et al., 2019).

Small molecule metabolites (< 1.5kDa) forming products of enzyme (coenzyme) mediated reactions consist of substrates, intermediates, signaling agents, and reactants (Dunn et al., 2011). Metabolites as biomarkers are a form of phenotypic expression present in organisms (human). These biological samples can be used to define biochemical mechanisms affected by environment, mental and physical stressors, and nutritional habits. Some of the first exercise-based metabolomics investigations using human athletes were first published using GC-MS platform in 2010 (Lehmann et al., 2010; Lewis et al., 2010). Lewis et al. (2010) measured >200 metabolites within several pathways pre- and post-exercise with plasma indicators. Many of the indicators belong to glycogenolysis, citric acid cycle, lipolysis, and niacinamide modulators of insulin. Duft et al. (2017) reviewed and compared 44 published exercise metabolomic studies consisting of four categories including metabolic responses to physical exercise, supplementation and physical exercise, sports performance, and physical exercise and disease. Of those studies,

17 used NMR and other platforms such as MS-GC spectrometry showing the benefits of oral supplementation lowering levels of CVD, and their potential for intervention in FF cardiovascular health.

Nuclear magnetic resonance spectrometry requires very little preparation time for different sample types such as biofluids. Blood plasma have been used in numerous studies to identify targeted or untargeted metabolites and their role in health factors such as CVD. Furthermore, NMR has been used to distinguish human disorders such as leukemia (MacIntyre et al., 2010) and other diseases. Research using this platform can be employed to monitor energy production, OS and antioxidant balances following nutritional supplementation and stress related activities. For example, Meisser et al. (2017) found that plasma levels of glucose, glutamine, alanine, and BCAA decreased after exercise at higher altitude causing protein pathways to become involved while lipid use was similar at sea level. HIIT protocols by Zafeiridis et al. (2016) reported that energy pathways from glycolysis and the citric acid cycle had the greatest metabolic changes during aerobic exercise. Whereas Danaher et al. (2016) using high intensity exercise (HIE) to simulate physiological remodeling reported significant metabolite changes post-exercise and at the end of recovery. Metabolite fingerprinting data from blood using ^{1}H NMR can be used to evaluate various diet protocols such as CRD by monitoring changes in metabolic homeostasis affecting energy levels, OS, or other imbalances by measuring concentrations of metabolites or their groupings. The stored blood plasma subsamples from the Waldman et al. (2020) 28-day-CRD provided a unique opportunity to increase the level of metabolic knowledge from the previous investigation attempting to reduce OS and improve cardiovascular health in FF (Waldman et al., 2020).

Objectives: Was to determine if ^{1}H NMR can be used to detect additional untargeted metabolites from stored blood plasma subsamples to further evaluate the 28-day-CRD for improving FF cardiovascular health, and to correlate specific up or downregulation of metabolites in their associated pathways to infer OS associated cardiovascular health.

Methods

Participants

In the investigation, 15 active male FF participants were selected based on criteria previously discussed (Waldman et al., 2020) following the American College of Sports Medicine (ACSM) low-risk guidelines (American Association of Sports Medicine, 2013). Additional details concerning participant eligibility can be found in the paper by Waldman et al. (2020) and the study approval by University's Institutional Review Board (IRB). The eligibility guidelines required that all subjects be cleared for duty by a physician, followed by a general health screening, dietary history and physical activity readiness questionnaires and medical history report. The initial screening participants had their body mass and height measured, body composition assessments for fat and lean muscle mass were done using the seven-site skinfold procedure. Following these assessments blood was drawn from each participant to evaluated baseline metabolic levels. Stored blood plasma samples from that the study and used in the current investigation, cannot be tied to specific individual participating FF.

Study Design

The study was established as a within-subject, repeated measures design containing 3 sessions across nine separate occasions (trials 1-9) consisting of a familiarization session (preliminary assessment trial 1-3), 15-day Western diet, baseline measurement session (4-6), 28-

day-CRD, and post-CRD (Trials 7-9; Waldman et al., 2020). Trials 4-9 included the experiment portion of the study determining body composition, physical performance, and metabolic analysis. Blood plasma sampling was conducted at the start of each of three sessions (Trials 1,4, and 7 (Waldman et al. 2020). The 3 sessions are as follows:

Session 1 **(Familiarization)**

Session 1 was designed eliminate any potential learning or order effects and consisted of trials 1-3 that were subdivided into 48-hour segments (Waldman et al., 2020). Blood samples were drawn before any activities at the start of trial 1 as described below. The session was used to measure body composition, metabolic and Wingate measurements, and FF physical performance. In the current study, metabolite data from the stored blood plasma was determine. At the conclusion of trail 3, a 15-day Western Diet was implemented prior to the start of session 2.

15-Day Western Diet

The 15-day diet was used in the study to establish baseline metabolic status for the FF participants. The 15-day diet also prepared the FF to prepare themselves for the specific exercise protocols, and food application methods to monitored caloric intake and food types prior to starting the 28-day-RCD plan implementation and exercise protocols.

Session 2 **(Baseline)**

Trials 4-6 were conducted following completion of the Western diet. As in session 1, just prior to start of trial 4, blood was drawn for targeted biochemical analysis. Following

completion of the blood draw, trial 4 was initiated as per Waldman et al. (2020) including body composition (discussed in Participants section), metabolic and Wingate measurements, and FF physical performance assessments. Blood plasma was drawn immediately following the 15-day Western diet that was conducted prior to start of trial 4 to obtain specific baseline metabolic data before the 28-day-CRD was initiated.

28-Day-Carbohydrate Restrictive Diet

The CRD was conducted after completion of Trial 6 and all details concerning the diet can be found in Waldman et al. (2020). The CRD diet used *ad libitum*, nonketogenic CRD designed to accommodate the occupant, lifestyle and eating patterns of the participants. The *ad libritum* CRD used in the study attempted to accommodate variable physical activity where carbohydrate intake was flexible within a certain range. Immediately following the diet protocol, Session 3 was implemented.

Session 3 (Post-CRD)

Following completion of CRD trials session 3 was conducted during trials 7-9. As in session 2, just prior to start of trial 7, blood was drawn for targeted biochemical analysis. After the blood draw, trial 7 was initiated that included body composition (see Participants section above), metabolic and Wingate measurements, and FF physical performance assessments. The study concluded after completion of trial 9.

Blood Sampling

Blood was sampled during trials 1, 4 and 7 of the three sessions with replicate plasma subsample ranging in volume from 1 to 3 ml stored at -80°C (Waldman et al., 2020). The participants fasted for 10 h prior to each sampling date, having refrained from alcohol and

caffeine consumption 24 h before each trial. After anthropometric measures were obtained, each subject was then instructed to lie quietly in a supine position for 5 min to minimize acute plasma shifts that occur when moving from standing to sitting (Harrison, 1985). Following this rest period, 12 ml of antecubital venous blood was drawn into two, 6 ml sodium heparin anticoagulant vacutainers via a 21 G, Safety-Lok butterfly needle (REF# 367287, Franklin Lakes, NJ). All sealed vacutainers were centrifuged at 4°C for 10 min at 2500 rpm, followed by plasma storage at -80°C for subsequent analysis. Following the 28-day-CRD diet, antecubital blood samples were drawn during session 3 using the same methodology as those completed in the previous sessions.

Plasma Preparation and Protein Removal

Samples of FF blood plasma was prepared using published methods protocol (Dona et al., 2014-S.3) and later with minor revision during filtration of blood plasma to remove excess glycerol and proteins (Rivera-Santos et al., 2021). Specifically, plasma samples stored at -80°C were thawed to room temperature. It was noted in preliminary runs that large proteins prevented accurate identification of metabolites. To overcome this problem, 3 kDa molecular weight cutoff (MWCO) Omega membrane Microsep Advanced Centrifuge Tubes (Pall Corp., Ann Arbor, MI) were used to remove large proteins from the plasma. To remove glycerol, the tubes were rinsed in ultrapure water, filled to capacity, and centrifuged at 3,800 relative centrifuge force (RCF) for 10 min at 20° C. Water was removed from both the retentate vial and the collection tube of the ultrafiltration unit, and centrifugation was repeated. After two rounds of centrifugation, the unit was emptied and thoroughly dried. This process was found to reduce glycerol present in the tube filters to prevent contamination and artificially increase plasma sample concentrations. Immediately following glycerol removal, 900 µL of a bovine plasma sample (described below)

was added to the concentrator and centrifuged at 3,800 RCF for 25 min at 20° C. The flow-through from the collection tube was stored into a vial at 4° C until ^{1}H NMR.

NMR Sample Preparation

A total of 48 blood plasma samples were analyzed using ^{1}H NMR spectrometry from the 15 FF participants comparing metabolites and concentrations associated with the CRD plan conducted during the three sessions (trials 1, 4, 7). Specific detailed of the methodology for conducting the NMR blood plasma preparation and analyses are detailed below.

Blood plasma samples were aliquoted with 330 µL of an NMR referencing stock solution and prepared as follows. The solution consisted of 200 mM sodium phosphate buffer, 1 mM 3-trimethylsilylpropionate 2, 2, 3, 3-d4 (TMSP-d4, Cambridge Isotope Labs DLM-48-5) and 0.1% (w/v) sodium azide (NaN_3) in 50% D_2O. 50 mL volume of referencing solution was prepared as follows: 0.7356 g NaH_2PO_4 (sodium phosphate monobasic, anhydrous 99%), 0.5492 Na_2HPO_4 (disodium hydrogen phosphate, anhydrous 99%), 50 mg NaN_3, and 8.613 mg of TMSP-d4 as an internal standard. Then 25 mL of ultrapure water was added, vortexed, pH adjusted to 7.0 using concentrated HCl or 1 M NaOH. The buffer solution was then transferred to a 50 mL volumetric flask, and 25 ml of deuterium oxide (D_2O, Cambridge Isotope Labs DLM-4-99) was added to the buffer solution, for a final volume of 50 mL which was thoroughly mixed. A 0.2 µM syringe filter was used to sterilize the referencing solution. The filtered plasma samples were mixed 1:1 with referencing solution and following mixing the entire 660 µL sample was transferred to clean NMR tubes (Wilmad LabGlass, 535-PP-7). The same solution was used for all samples described in this study to ensure uniformity during the experiment.

NMR Spectroscopy

Samples were analyzed with a Bruker Avance III-HD 500 MHz spectrometer equipped with a BBFO Probe (Bruker, MA, USA). Samples were run under automation mode using a SampleJet autosampler, with all samples refrigerated at 4 °C until just prior to analysis. Data was acquired using a perfect-echo WATERGATE sequence (PE-WATERGATE, parameter set ZGESGPPE) (Adam et al., 2012) for 128 scans with an inter-scan delay of one second and a per-scan acquisition time of 3s. Including 3D-shimming, the total time of acquisition for each sample was approximately 17 minutes. Raw spectra were imported into TopSpin 4.0.8 (Bruker Corporation, Bilerica MA), automatically phased, baseline corrected, and referenced to the TMSP at 0 ppm. Processed spectra were then imported into Chenomx NMR Suite (Chenomx, Edmonton, Canada) software to identify individual metabolites and relative concentrations using the 500 NMR reference library and the Human Metabolomic Database (HMDB.ca).

Statistical Analyses

The follow statistical analyses were used to compare metabolite diversity and concentrations. Initial univariate analysis data were subjected to analysis of variance (ANOVA) using a crossover design with SAS® PROC GLM (SAS/STAT ver. 9.4). Means were separated using Fisher's Least Significant Difference (LSD). The analysis compared the metabolites and their concentrations between session 1 (trials 1-familiarization), session 2 (trial 4-pre CRD and session 3 (trial 7 post CRD). Statistical significance was set at $P \leq 0.05$ and ANOVA values are expressed as means ± SE (standard error). Because more than two variables were compared for this study analysis, fold change and volcano plot consisting of fold change and t-test comparisons could not be conducted.

For multivariate analyses, all metabolite concentrations were analyzed using MetaboAnalyst 4.0 platform (https://www.metaboanalyst.ca). Binned data was preprocessed applying normalization, data transformation and pareto scaled. Data was normalized by sum to minimize the differences in concentrations of the plasma samples.

Multivariate statistical analyses were conducted included principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA; Westerhuis et al., 2010, Sun et al., 2014). Principal components analysis (PCA) was conducted to obtain a score plot that shows percent variation of the test variables. Results were used to determine predefined groups and remove metabolite concentrations. Scores are weighted average of the original variables and weighted profiles are called loadings. The partial least squares discriminant analysis (PLS-DA) method uses supervised multivariate regression to obtain linear combinations of original variables (X) that can predict associations of metabolites into groups (classes). These statistical methods define, or measure class discrimination based on prediction accuracy and separation distance based on the ratio between and within the group sum of squares (B/W ratio).

Two key variables to measure model predictive ability (goodness-of-fit) were cross-validation using Q^2 values and permutation rate (significant if $p \leq 0.05$. Specificity, sensitivity, and classification rates (percentage samples correctly classified) were obtained within a 1000-fold cross-validation for PLS-DA and OPLS-DA models. In those models the Q^2 parameter represents the predictive ability of the model supporting the authenticity of the results and permutation is used to validate and test the degree of over-fitting of the model (Sun et al., 2013). The OPLS-DA model maximized the covariance between measured data (X-variable data, concentrations from NMR spectra) and (Y-variable, predictive). Score plots combining the

correlation from OPLS-DA models were used to distinguish differences in concentrations among groups. Cross-validation was used to verify if the model differentiated metabolite concentrations.

If the models were positively crossed-validated for PLS-DA and OPLS-DA, then the Variable Importance in Projection (VIP) analysis could be used for individual metabolite discussion. VIP is a weighted sum of squares associated with amount of Y-variation explained for each dimension. This is a weighted sum of PLS-regression for PLS-DA and OPLS-DA. Each metabolite (variable) with a VIP weight > 1.0 from the analyses was determined have most variation in concentrations (Cho et al., 2008). Using VIP greater than 1 is considered an acceptable cutoff threshold to define relevant variables (metabolite concentrations) using PLS regression or linear classification model (Akarachantachote et al., 2014). The PLS-DA and OPLS-DA are supervised models that are used to indicate variations in metabolite datasets to predict groups or clusters of metabolite concentrations treatment effects.

Results

A total of 40 metabolites were identified from NMR spectra during the familiarization, pre- and post-CRD blood sampling sessions (Table 1). An additional 7 metabolites not listed in the table including 2-hydroxybutryate, arginine, choline, cysteine, erythritol, pyroglutamate, and 3-methylhistidine were inconsistently detected by NMR. ANOVA compared mean concentrations for each metabolite between the three sessions. The analysis showed that 8 metabolites were significantly ($p \leq 0.05$) upregulated and 3 downregulated during the 28-CRD. These 11 compounds were β-hydroxybutyrate, acetate, acetone, alanine, betaine, glycine, histidine, isoleucine, leucine, urea, and valine. Alanine, betaine, and histidine were the only metabolites that were significantly downregulated between familiarization and pre-CRD blood

samplings. The remaining 37 metabolites were similar between the first two sampling periods indicating overall metabolic homeostasis prior to the 28-day-CRD challenge.

When comparing the data across the three sessions, the ketone bodies acetone and β-hydroxybutyrate were significantly upregulated from pre- to post-CRD which is indicative of lipolysis induced hormonal action (lower insulin levels) from the limited carbohydrate intake (Sikder et al., 2018). The missing ketone body in the current study acetoacetate is unstable and the metabolites levels drastically drop within the first 1-2 h. It has a maximum in plasma a half-life occurs at 0.8 to 3.1 h after blood draw. This prevents accurate quantification in NMR studies (Clarke et al., 2012). Even though the levels of the two ketone bodies increased post-CRD they remained within the expected normal blood range and never reached levels of ketosis in any of the participants.

The branch chain amino acids (BCAAs) isoleucine, leucine, and valine significantly upregulated post-CRD (Table 1). The increases in concentration remained within the normal range of blood metabolism but these concentrations show CRD treading towards improved metabolism. Other amino acids formed from catabolism of BCAAs such as glutamine and glutamate were both numerically similar as was the metabolites glucose, lactate and pyruvate associated with energy metabolism throughout the entire study.

The PCA model derived from the ^{1}H NMR spectra for all samples was applied to the full data set for three sessions (trials 1, 4 and 7) in which first two principal components cumulatively accounted for 30.9% of the data variation explained as shown in the spree plot (Figure 1). Principal component 1 accounted for 20.4% of the total variation and component 2 was 10.5%. However, separation of metabolite concentrations between the three sessions could

not be clearly determined as principal components 1 to 5 accounted for 54.5% (Figure 2). To increase visualization of the metabolic data, a PLS-DA was performed. The best separation among the CRD was made by the supervised model PLS-DA components 1 and 2 contributing to 27.6% of the variation (Figure A1). Even though there was a slight trend toward increased concentrations of the metabolites following the 28-day-CRD, the model was not supported using the cross-validation indicators (Q^2 = - 0.082 and permutation test (1000 times) with p = 0.30). Cross-validation indicators from OPLS-DA did not support the model as Q^2 = 0.060 and permutation test p = 0.30 (Figure A2).

Figures from multivariate analyses for PLS-DA and OPLS-DA models that were not supported using cross-validation criteria can be found in Appendices A. These include Figures A1-A10 for the models comparing treatment effects on metabolite concentrations clusters.

Table 1

List of Metabolomes from Blood Plasma Collected at Three Time Periods Pre and Post 28-day Carbohydrate Restricted Diet

	Session 1 Start of Familiarization Protocol	Session 2 Pre-Carbohydrate Restricted Diet	Session 3 Post-Carbohydrate Restricted Diet	P-value/SE
Metabolites		Concentrations mM [a] Means	Concentrations mM Means	$p \leq 0.05/ \pm$ SE
2-Aminobutyrate	0.086	0.031	0.084	0.662/0.015
2-Hydroxyisovalerate	0.009	0.010	0.027	0.137/0.002
2-Hydroxyvalerate	0.071	0.054	0.089	0.403/0.007
β-Hydroxybutyrate	**0.011B[b]**	**0.007B**	**0.063A**	**0.238/0.008**
4-Aminobutyrate	0.037	0.031	0.033	0.024/0.006
Acetate[a]	**0.036AB**	**0.031B**	**0.045A**	**0.032/0.018**
Acetone	**0.013B**	**0.014B**	**0.038A**	**0.006/0.003**
Alanine	**0.410A**	**0.327B**	**0.328AB**	**0.034/0.013**
Ascorbate	0.056	0.009	0.024	0.057/0.016
Asparagine	0.086	0.060	0.126	0.671/0.021
Betaine	**0.146A**	**0.094B**	**0.171A**	**0.003/0.008**
Carnitine	0.061	0.033	0.066	0.568/0.007
Citrate	0.027	0.013	0.033	0.533/0.008

Table 1 (Continued)

Creatine	0.019	0.034	0.034	0.810/0.006
Creatinine	0.104	0.089	0.105	0.978/0.007
Formate	0.007	0.011	0.014	0.675/0.002
Glucose	2.883	2.877	2.778	0.570/0.207
Glutamate	0.040	0.060	0.092	0.230/0.011
Glutamine	0.552	0.448	0.528	0.819/0.029
Glycerol	1.492	1.511	1.770	0.264/0.063
Glycine	**0.121B**	**0.163AB**	**0.261A**	**0.111/0.026**
Histidine	**0.072A**	**0.027B**	**0.053A**	**0.006/0.005**
Isoleucine	**0.074B**	**0.058B**	**0.105A**	**0.002/0.004**
Lactate	0.996	0.801	0.821	0.209/0.050
Leucine	**0.152B**	**0.120B**	**0.197A**	**0.0050.008**
Lysine	0.112	0.113	0.139	0.695/0.019
Mannose	0.056	0.060	0.376	0.451/0.066
Methionine	0.025	0.018	0.023	0.702/0.002
N,N-Dimethylglycine	0.004	0.003	0.005	0.458/0.005
Ornithine	0.016	0.019	0.061	0.253/0.009
Phenylalanine	0.011	0.017	0.015	0.942/0.003
Proline	0.155	0.145	0.133	0.954/0.011
Pyruvate	0.031	0.023	0.031	0.726/0.005
Sarcosine	0.004	0.001	0.007	0.775/0.002
Serine	0.094	0.012	0.265	0.712/0.062
Threonine	0.031	0.045	0.086	0.859/0.021

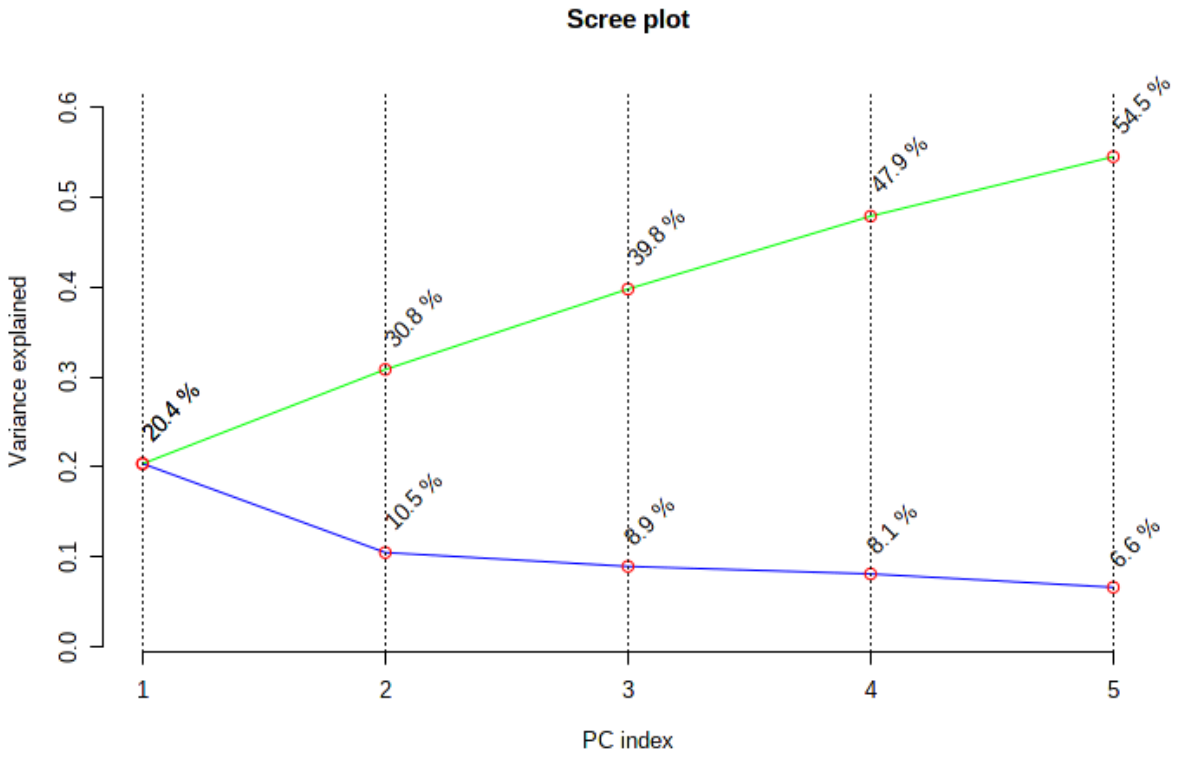
Table 1 (Continued)

Tyrosine	0.039	0.029	0.046	0.248/0.004
Urea	**4.811B**	**3.945B**	**8.948A**	**0.0440.791**
Valine	**0.182AB**	**0.140B**	**0.238A**	**0.050/0.015**
1-methylhistidine (Pi)	0.019	0.036	0.0.39	0.433/0.006

[a] Numbers represent mean metabolite concentrations mM. [b]Mean concentrations followed by same letter were significantly different ($p \leq 0.05$) according to Fishers LSD.

Figure 1

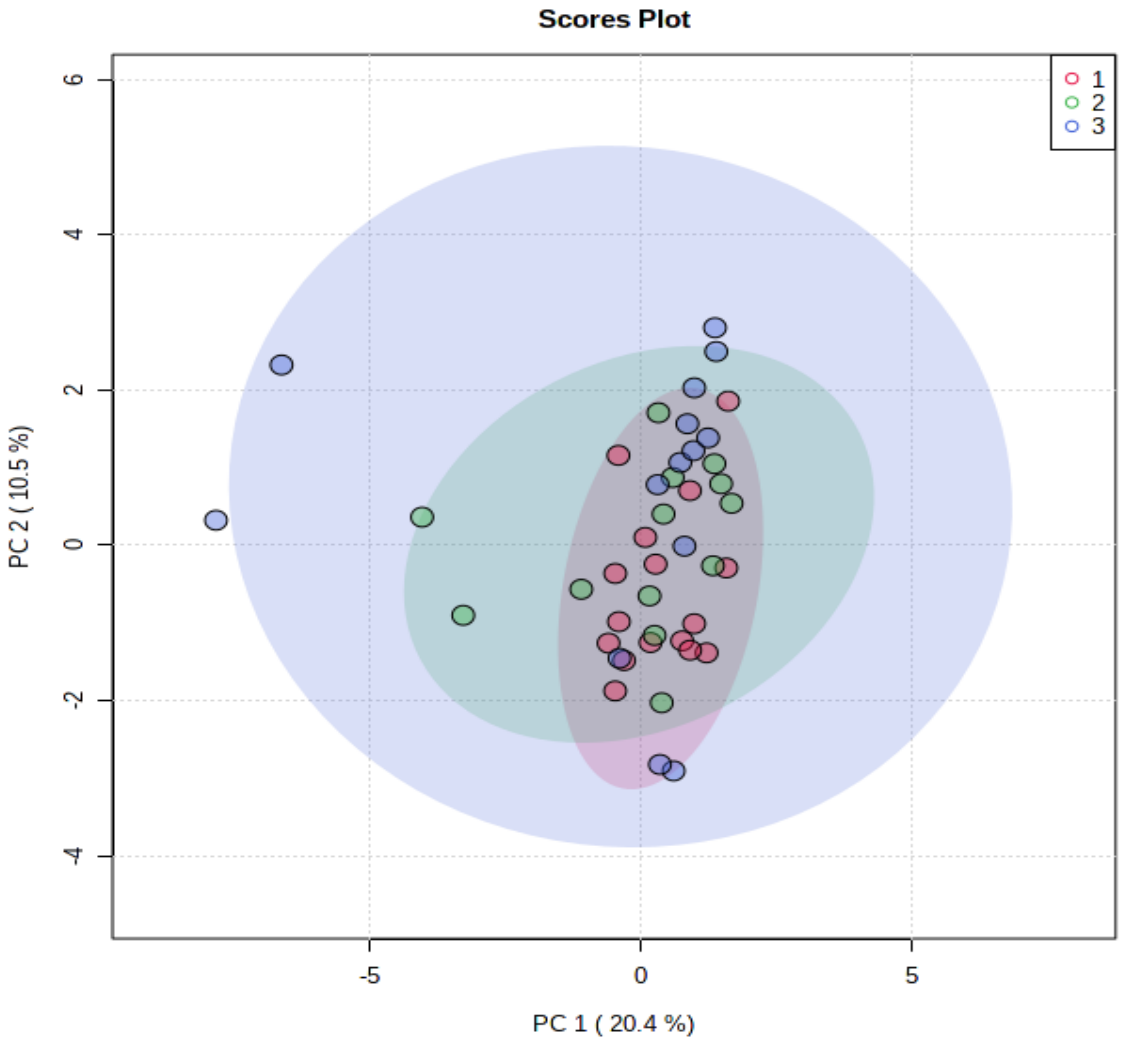
Spree Plot for Principal Component Analysis (CRD)



Note: Total variations from PCA modeling of metabolite concentrations blood plasma sampling prior to three sessions (trials 1, 4 and 7). The spree plots compared variance of 5 variables with first two components with a low total variation of 30.8% not supporting the model.

Figure 2

PCA Score Plot (CRD)



Note: Metabolite concentration distributions for the three sessions (trials 1, 4 and 7) of firefighter's blood plasma with % variance in brackets. Session 1= familiarization, 2= baseline, and 3= post-28-day carbohydrate restricted diet.

Discussion

A total of 40 consistently detectable metabolites (7 inconsistently) were identified from the FF blood plasma following long-term storage using ^{1}H NMR. The metabolites detected in this study are the same as those listed in previous blood plasma studies (Gowda & Raftery, 2014; Gowda et al., 2015). It was reported that ~67 metabolites could be quantified using a serum/plasma-based metabolomics approach (Gowda et al., 2015) but another study suggested that intact serum/plasma were limited to ~30 detectable metabolites (Psychogois et al., 2011). It is believed that plasma/serum proteins quickly bind up the metabolites preventing their detection suggesting it is best to expedite metabolomic analysis. In the current study, the available volumes of the stored blood plasma subsamples limited analyses to the conventional human blood plasma metabolomics extraction protocol for ^{1}H NMR described above. However, other important metabolites not detected in this study such as lipids, major coenzymes, and important antioxidants, require different sample preparation protocols using whole blood for ^{1}H NMR analyses (Lin et al., 2009; Gowda & Raffery, 2014; Gowda & Rafferty, 2017; Duft et al., 2017; Deidda et al., 2017). Whole blood provides the best option for greater quantification of important biomarkers directly associated with monitoring cellular functions impacting OS and cardiovascular health. Metabolites such as the coenzymes nicotinamide adenine dinucleotide (NAD) and the antioxidants in the glutathione group are unstable but can be detected using whole blood (Gowda & Rafferty, 2017). Since many important metabolites are not stable following sampling the speed of conducting ^{1}H NMR analysis following blood sampling is critical.

Metabolite Determination

Unique to omics studies, Chenomx programing can now evaluates chemical shifts within each sample providing metabolite identity though their libraries and interconnected online resources. Another important feature is that Chenomx program detects changes in metabolite concentrations based on treatment effects that then can be compared using univariate and multivariate analyses. The Chenomx program coupled with HMBD provide a strong approach for identify metabolites and their concentrations obtained from ^{1}H NMR studies.

Short and Long-Term Storage

Metabolite concentrations vary based on factors such as nutrition supplementation, physical stressors, human disease, and timing of actual analytical testing following sampling. The blood plasma samples in the current study were stored up to 24 months prior to analysis. Short-term and long-term storage in -80°C can confound the detection and concentrations of metabolites but long-term levels of the metabolites did not occur until 7-years after sampling (Wagner-Golbs et al., 2019). For short-term storage it was reported that lipid compounds significantly changed in 2.5 h at room temperature and during freeze-thawing followed by NMR analyses (Pinto et al., 2014). When −20 °C and −80 °C EDTA plasma samples were compared after 1 month storage the metabolite data was similar. However, there were small decreases in lipids and acetone but increases in choline compounds, alanine, glucose, and pyruvate. In the current study alanine did have a transient increase but glucose and pyruvate were similar.

For long-term storage, EDTA plasma samples stored for 7 years showed that 2% of the metabolites were altered especially concentrations of fatty acids with greater sensitivity by the amino acids (Wagner-Golbs et al., 2019). The glycolytic component pyruvate and the amino acids asparagine, cysteine, and cystine were significantly reduced in concentrations while

aspartate increased. In another study plasma metabolic changes were negligible after 30 months of storage at −80°C (Pinto et al., 2014). Based on those previous studies, plasma and serum sample metabolites remained stable from 30 month to 7-years (Wagner-Golbs et al., 2019) and storage should not have affected the current studies results.

Univariate Analyses

In the current study, glucose, pyruvate, and lactate that are associated with various metabolic processes, were similar between the session including pre- and post-28-day-CRD. Mean overall concentrations of glucose from ^{1}H NMR analysis were lower than normal for the FF blood plasma. The normal range was reported to be between 4 to 5 mM/L in human plasma (Clark et al., 1990) while the overall mean for the participants averaged ~ 3.5 mM/L. A clinical study reported that unprocessed glucose in a laboratory can decrease between 5-7% per h (Mikesh & Bruns, 2008). Also, loss of glucose in plasma can be 5% per h but up to 40% in 3 h due to continued activity of glycolytic enzymes (McMillin 1990; van‘t Erve et al., 2014). Based on the literature, mean glucose concentrations in the current study can be attributed to early changes rather than from long-term storage.

Following the 28-day-CRD challenge, none of FF were in ketosis or subketosis (Waldman et al., 2020). Glucose levels in the current study were similar during the three sessions suggesting homeostasis was maintained but concentrations of the two ketone bodies significantly increased following the diet. β-hydroxybutyrate and acetone significantly increased following the 28-day-CRD but were similar during familiarization session 1 and pre-CRD for session 2 (Table 1). As ketone levels increase, insulin deficiency can occur causing glucagon formation initiating lipolysis of triglycerides from adipose tissues. This suggests that the catabolism of triglycerides from adipose tissues may occur for supporting homeostasis of blood

glucose levels, and free fatty acids catabolism for energy production contributing to weight loss by the FF during the previous study (Waldman et al., 2020). Adipose tissues act as endocrine organs that secrete proinflammatory cytokines such as IL-6 and TNF-α, macrophage, and T cells supporting downstream chronic inflammation and free radical formation causing OS. Increased chronic obesity contributes to atherosclerosis causing metabolic syndrome (Nishimura et al., 2009) and may trigger sudden death in FF if atherosclerosis levels are severe in an individual especially during stress related activities.

The BCAA isoleucine, leucine, and valine were significantly greater using ANOVA following the 28-day-CRD than the pre-CRD sessions. Previous studies have reported that BCAA supplementation before and after exercise decrease muscle damage and inflammation while promoting protein synthesis supporting reduce OS (Holeck, 2018). The activation of protein synthesis via mTOR signaling pathway by BCAA (mainly leucine) were implicated in improved insulin resistance, glucose transport, inducing mitochondrial biogenesis, and as signaling molecules for oxidative stress defenses (D'Antona et al., 2010). Furthermore, it was reported that isoleucine has a hypoglycemic effect associated with increased glucose uptake in skeletal muscles and decreased gluconeogenesis in liver (Doi et al., 2007). Furthermore, BCAA are catabolized during protein synthesis forming glutamate an intermediate in the formation of antioxidant glutathione (Siddik & Shin, 2019). Even though BCAA increased following 28-day-CRD their overall concentrations remained within the normal range for blood plasma.

While most amino acids are synthesized in the liver, BCAAs which are stored primarily in skeletal muscles are generally catabolized following exercise or if blood glucose levels are reduced during the CRD (Holecek, 2018). The other source of BCAA during the study was from the exogenous increase in protein consumption by the FF during the 28-day period. With the

need for ATP production for energy to be maintained the BCAA can be converted to the final products acetyl-COA and succinyl-COA for use in the citric acid cycle or for protein synthesis to repair damaged tissues following exercise (Mitch & Golberg, 1996; Holecek, 2018).

Another metabolite significantly upregulated following 28-day-CRD and indirectly associated with reducing OS is β-hydroxybutyrate. With lower glucose availability for glycolysis during food deprivation or prolonged exercise, β-hydroxybutyrate is synthesized in the liver from fatty acids. The metabolite is converted back to acetyl-CoA during glucose deficits leading to use in the citric acid cycle and to electron transport chain supporting oxidative phosphylation for ATP enhancing oxygen consumption thereby suppressing ROS (Newman & Verdin, 2017). It was suggested that β-hydroxybutyrate mediates complex II of electron transport chain improving mitochondrial respiration resulting in lower ROS while NADH drives complex I (Tieu et al., 2003). Also, β-hydroxybutyrate initiates downstream signaling for cellular repair and gene expression, and lipid metabolism (Newman & Verdin, 2017). β-hydroxybutyrate has a signaling role as an endogenous inhibitor of histone deacetylases and can be a ligand for cell receptor inhibiting lipolysis associated with insulin production (Boden, 2011; Newman & Verdin, 2014). Other metabolic products produced downstream are succinyl-CoA and coenzyme NAD^+ which is critical to many metabolic processes for mitochondrial and cellular health.

In the current study, acetone and β-hydroxybutyrate were greater following the 28-day CRD. The diet leads to reduced exogenous sugar and carbohydrate intake requiring alternative metabolic coverage. To support glucose homeostasis during the CRD, possibly triglycerides primarily from adipose tissues are reduced to free fatty acids and glycerol. The free fatty acids are transported into the liver, β-oxidation occurs in mitochondria and are then converted to

acetyl-COA for either energy needs or forming acetoacetate, acetone, and β-hydroxybutyrate (Nie et al., 2018; Han et al., 2020)

Multivariate Analyses

Multivariate analysis using an unsupervised PCA model did not provide adequate metabolite group visualization and interpretation between the familiarization, pre-28-day-CRD, and post-28-day-CRD sessions. Percent variation of the first five principal components was low overall and specifically components 1 and 2 from score (spree) plots did not provide better visualization based on low total percent variations that were accounted for in the model. The supervised PLS-DA and OPLS-DA models were not supported either with cross-validation using Q^2 coefficient and permutation value results (Gowda et al., 2008, Sun et al., 2014). The VIP comparisons of the individual metabolites could not be used since the two supervised models were not supported preventing individual metabolite comparisons. The metabolite concentration data with limited replication may not be adequate to run effectively with multivariate analyses but may be more suited for NMR chemical shift spectral data (Gowda et al., 2008).

Past and Current ^{1}H NMR Study

The results of the Waldman et al. (2020) study determined that several metabolic markers may favor positive cardiovascular health in the FF after the 28-day-CRD. Glucose was the only biomarker that was previously evaluated, and the current study used the same blood plasma subsample for NMR. Even though the glucose challenge was given to the FF prior to blood sampling during session 2 (trial 4) and after 28-day-CRD for session 3 (trial 7) no significant concentration changes in plasma glucose levels occurred in the past or present studies suggesting insulin resistance was not a contributing factor to OS.

Conclusion

In conclusion, the majority of the metabolites that were detected in this study were the same as those identified in other investigations using blood plasma with a similar extraction protocol. The metabolites, and concentrations from three sampling times (sessions) could not be differentiated using multivariate analysis models PCA, PLS-DA or OPLS-DA based on low Q^2 coefficient values and/or permutation results, and overall low percent total variations were observed in components 1 and 2 for all three models. Thus, VIP scores for the individual metabolites could not be compared in this study. Furthermore, metabolite concentrations for multivariate analyses seemed to have limited applications for visualizing treatment effects but small a sample size may be partially responsible. It was determined that age of the samples stored at -80°C should be stable for majority of metabolites from plasma, for several years.

Metabolites have specific or multiple roles in human metabolism. An example of an indirect biomarker for reducing OS is the ketone body β-hydroxybutyrate which suppresses histone deacetylase activity (Kong et al., 2017). By this suppression, enhanced mitochondrial respiration is maintained preventing increased ROS formation as reported for mice (Monsalves-Alverez et al., 2020). The BCAA involved in protein synthesis such as leucine caused by activation of mTORC1, is also involved as a signaling molecule for OS defenses (D'Antona et al., 2010) and acts as a nitrogen donor involved in improved cellular functions and nitrogen shuttling (Sperringer et al., 2017).

Results of our study using ^{1}H NMR had slightly greater numbers of metabolites than previously investigations using blood plasma, but total metabolites detected were limited by the protocol, volume of blood plasma available, type of blood, and age of the samples. It is suggested for future OS studies that whole blood, serum and blood plasma be used to maximize

the detection of targeted and untargeted general metabolites using several sample preparation buffer protocols. More stringent timing protocols for ^{1}H NMR could enable direct metabolite antioxidant assessments of treatments impacting OS. This data could be evaluated to identify important targeted metabolites simultaneously as in the original investigation (Waldman et al., 2020).

CHAPTER IV

^{1}H NMR ANALYSIS ON IMPACT OF CURCUMIN SUPPLEMENT ON OXIDATIVE STRESS BIOMARKER IN PROFESSIONAL FIREFIGHTERS PERFORMING STRUCTURAL FIRE EXERCISES

Abstract

Firefighters (FF) are reported to have higher than normal levels of oxidative stress (OS) induced cardiovascular disease caused by occupational stress, low physical activity, and poor nutrition. In a previous investigation using FF, curcumin supplementation was ingested attempting to offset transient oxidative stress (OS) during training following a search and clear physical activity with heat stress protocol while wearing personal protection equipment (PPE). Results showed no benefit with curcumin or from simulated heat on transient OS following exercise. Exercise did support reductions in levels of OS as indicated by two targeted metabolic biomarkers from a previous investigation. Remaining blood plasma subsamples from that study were stored at -80° C for two years and reevaluated using ^{1}H NMR spectrometry to quantify and qualify untargeted metabolites that directly or indirectly impact transient OS. A total of 34 metabolites were consistently detected from the plasma samples and 10 others were detected intermittently. The metabolite concentrations of the 34 were analyzed with ANOVA between pre- and immediately post-exercise and 30 minutes post-exercise and were also compared between curcumin and a placebo. Lactate significantly upregulated immediately post-exercise while never reaching metabolic acidosis levels and returned to pre-exercise levels within 30 min

post exercise. Upregulation of lactate has been reported to initiate a homeostasis response mechanism promoting a mild reactive oxidative species (ROS) signaling causing antioxidant defense activation. When curcumin supplementation was compared to the placebo for each metabolite, urea and pyroglutamate were significantly downregulated with curcumin, but the two metabolites remained within normal blood plasma levels. Increased urea levels are reported to enhance OS while its reduction following curcumin ingestion suggests the need for further study to lower transient OS. Pyroglutamate is an intermediate of glutamine and glutamate, but no impacts can be noted based on the current data.

To visualize the results, multivariate analyses were conducted using principal components analysis (PCA), partial least squares-discriminate analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) models comparing metabolite concentrations. PCA was unable to distinguish the metabolite concentrations between the three blood sampling times and curcumin versus placebo. Cross-validation of PLS-DA and OPLS-DA using Q^2 coefficient, and permutation values did not support the models' comparing times of sampling or between curcumin supplementation versus placebo. Since the models were not supported individual comparisons between metabolites using VIP could not be presented.

In conclusion, lactate significantly upregulated following physical activity using the simulated heat protocol. Lactate is known to support signaling for antioxidant synthesis and may have been a factor in reducing transient OS. Even though two other metabolites were significantly downregulated with curcumin supplementation, no apparent trends were noted impacting redox levels. Furthermore, OS research regardless of oral supplementation type must include data on lipids, important coenzymes, and antioxidants requiring individualized ^{1}H NMR

protocols using whole blood versus plasma for determining their OS benefits for chronic stress management.

Introduction

Cardiovascular disease (CVD) has multiple causes that form a complex called metabolic syndrome (Waldman et al., 2020). First responder personnel such as firefighters (FF) are reported to have highest occupational risk of death due to CVD associated occupational oxidative stress (OS) (Huang et al., 2013; McAllister et al., 2018, 2019). Stressors increase OS leading to atherosclerosis include chronic physiological stress from occupational duties, environmental stressors from heat and smoke, emergency intense physical activities, nutritional, and erratic sleep behavior from living at a station (Dimsdale, 2008). Metabolic changes from the stressors can be monitored using intermediate metabolites such as those involved in formation of the catecholamine complex (Meiser et al., 2013).

Metabolic syndrome or syndrome X is defined as a cluster of risk factors associated with the development of CVD (Alberti et al., 2009). If these risk factors remain unresolved, they can lead to atherosclerosis causing ischemia and possible heart failure. The main factors associated with metabolic syndrome include hyperglycemia, high levels of low-density lipoproteins (LDL) and high triglycerides, hypertension, insulin resistance, and endothelial dysfunction that are usually associated with obesity (Alshehri, 2010). Of these it was suggested that common risk factors such as the elevated blood pressure, high plasma glucose, and atherogenic dyslipidemia contribute to prothrombotic and chronic proinflammatory states. These negative metabolic factors can occur from imbalances in redox signaling molecules from higher levels of reactive oxidative species (ROS) versus antioxidants concentration levels (Cornier et al., 2008). These high ROS metabolic states can be naturally modified or prevented in high risk first responders

including FF. Since transient OS stress occurs during moderate or normal physical activity, overall ROS levels can be reduced using acute or chronic nutrient supplementation coupled with regular exercise regimes (McAllister et al., 2019). These modifications can result in positive health changes reducing the damage from high ROS levels (Grundy et al., 2005; Alshehri, 2010; Kaur, 2014). These underlying causes of inflammation from OS are primarily associated from poor nutrition and low aerobic exercise activities leading to increased visceral fat obesity.

To improve cardiovascular health and education for FF, several studies were conducted that compared nutritional supplementations and exercise (McAllister et al., 2018, 2019; Waldman et al., 2020). Exercise is known to increase transient OS from ROS that support cellular signaling necessary for muscular adaptations (Powers et al., 2020), and reduced hypertension with increased cellular repair signaling for remodeling of damage cells (Radak et al., 2013). Using FF as participants, curcumin, ketone salt supplements or carbohydrate restrictive diet with exercise protocols showed anthropometric benefits, and reduced OS trends associated with lowering CVD risks (McAllister et al., 2018, McAllister et al., 2019 and Waldman et al., 2020).

Exercise is an important mitigator of CVD and has many other health related benefits, thus physical activity should be part of our lives (Kullo et al., 2007). Specific metabolites such as glucose, ketone bodies and other intermediates are used to monitor changes when exercise metabolic changes occur (Duft et al., 2017). As with any form of stress related activity, transient ROS will increase but intensities of the exercises that can cause increased inflammation and OS (Park & Kwak, 2016; Ji et al., 2016; Powers et al., 2020). Exercise induced OS is transient and very important for metabolic signaling to support positive cellular functions (e.g., mitochondrial)

and adaptations resulting in muscular remodeling and hypertrophy (Ji et al., 2016; Powers et al., 2020).

When mitochondria dysfunction occurs from formation of ROS along the electron transport chain increased levels of ROS and enzymes such as xanthine oxidases and NADPH oxidase has been observed (Zorov et al. 2014). Eccentric exercise stimulated transient myostatin mRNA expression inhibiting myogenesis for up to 24 h post exercise (Koska et al., 2000). Eccentric muscle exercise protocol plus a curcumin supplementation blunted creatine kinase, and muscle soreness ratings were lower possibly indicating lower muscle damage (Bashham et al., 2019). Furthermore, antioxidant capacity, malondialdehyde, and TNF-α did not show any difference between the curcumin supplementation and placebo groups. In a study evaluating exercise and nutritional supplementation to reduce CVD using FF blood plasma, OS markers such as SOD, H_2O_2, and AOPP 30- and 60-min post exercise were significantly lower. This is an indication of the benefits of heat associated physical exercise with a curcumin dietary supplementation (McAllister et al., 2018). Analysis of available blood plasma subsamples from the latter study would enable newer analytic methods such as a ^{1}H NMR platform to obtain additional metabolite data to further define the impacts of the exercise and curcumin nutrition on possible OS.

Curcumin, a naturally occurring spice found in rhizomes of turmeric (*Curcumin longa*) plants is a polyphenol with a chemical composition of bis-α, β-unsaturated β-diketone, named (E, E)-1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5 dione (Milobedeska et al., 1910; Hatcher et al., 2008). It is known to be a nutritionally safe pharmacological supplement containing antioxidant, anti-inflammatory, antiarthritic, antiviral, and anticancer properties (Srimal et al., 1973; Sugiyama et al., 1996). Curcumin has been reported to provide benefits for

reducing OS associated with inflammation muscle damage and muscle soreness (McAllister et al., 2018; Basham et al., 2019). Specifically, curcumin regulates various molecular targets including transcription and growth factors, inflammatory cytokines, protein kinases and other select enzymes with potential metabolites that can be used to monitor cellular health (Zhou et al., 2011). Curcumin having hypolipidemic properties through AMPK activation, suppresses fatty acid and cholesterol biosynthesis including fatty acid synthase and HMG-CoA reductase supporting fat catabolism and balanced OS:antioxidant ratio levels (Liu et al., 2017). Curcumin may enhance or inhibit many different enzymes to either activate or prevent different metabolic functions generally all resulting in health benefits which can reduce OS and CVD. However, results from many curcumin supplementation studies are inconclusive or show no benefits.

The possibility of using metabolomics in human nutrition and exercise related fields to define treatment benefits are becoming increasingly common with many different analytical platforms such as Gas Chromatography-Mass Spectrometry (GC-MS) and NMR to monitor changes in metabolites and concentrations (Duft et al., 2017; Sakaguchi et al., 2019). A review paper consisting of 24 studies evaluated metabolic responses to exercise across several analytical platforms such as NMR and types of univariate and multivariate analyses of the data (Sakaguchi et al., 2019). An acute exercise study which compared workload between recreational and soccer athletes reported modest shifts in metabolites (Zafeiridis et al., 2016) compared to high intensity interval exercises (HIIE) using ^{1}H NMR analysis of plasma. Results of the study indicated upregulation of carbohydrate-lipid metabolism and the citric acid cycle following short and long-interval exercise. Messier et al. (2017) determined that prolonged exercise at altitude caused a decrease in blood plasma levels of glucose, glutamine, alanine, and branched-chain amino acids (BCAAs) involving a protein pathway while lipid use was similar at sea level using NMR.

Danaher et al. (2016) using high intensity exercise (HIE) to simulate physiological remodeling reported significant metabolite changes post-exercise and at the end of recovery. In the study, significant metabolite differences were noted for lipids such as cholesterol, and lactate from glycolysis was more pronounced during recovery.

Duft et al. (2017) compared 44 published exercise metabolomic studies that included physical exercise and supplementations using various metabolome platforms including NMR. A milk supplementation containing whey protein and calcium caseinate ingested before a strength 1 RM protocol reported a dynamic plasma metabolic change in levels of alanine, β-hydroxybutyrate, branched amino acids, creatine, glucose, glutamine, glutamate, histidine, lipids, and tyrosine post-exercise (Yde et al., 2013). Using a ^{1}H NMR platform, Miccheli et al. (2009) evaluated effects of isotonic green tea sport drink on recovery after strenuous and prolonged exercise with a row ergometer on the metabolic profiles from plasma and urine biofluids. The analysis showed an effect to glucose, citrate, and lactate levels from plasma and for acetone, β-hydroxybutyrate, and lactate in urine. The ^{1}H NMR platform can potentially provide large numbers of metabolic biomarkers from biofluids and tissues that can contribute to research on energy production, disease diagnostic information, and impacts of stress factors associated with OS leading to CVD.

In the original study evaluating curcumin and exercise with heat, several targeted metabolic biomarkers supported reduced OS markers following exercise from blood plasma samples (McAllister et al., 2018). Specifically, glutathione, catalase, and advanced oxidation protein products (AOPP) activity were significantly greater immediately after exercise but returned to pre-exercise levels within 30 min post exercise. With the availability of stored blood plasma subsamples at -80°C from that study, other methods should be considered to further

evaluate potential benefits from curcumin supplementation and exercise on OS levels. With the availability of ^{1}H NMR platform, evaluation of FF blood plasma subsamples could provide information on the benefits of curcumin and exercise for reducing transient OS contributing to cardiovascular disease. The stored blood plasma subsamples from the McAllister et al. (2018) curcumin supplementation study provided a unique opportunity to increase the level of metabolic knowledge from the previous investigation attempting to reduce OS and improve cardiovascular health in FF (McAllister et al., 2018).

Objectives: To determine effects of an acute curcumin supplementation on transient OS caused by artificial heat stress with physical activity on metabolic composition of stored blood plasma using ^{1}H-NMR. Furthermore, to determine if metabolic concentrations can provide direct or indirect biomarker information supporting reduced OS and improving cardiovascular health as suggested in the previous published study.

Methods

Methodologies and procedures for this study evaluating nutritional supplementation protocol impacts and exercise induced OS were conducted at Mississippi State University follows those as discussed previously (McAllister et al., 2018). Ten healthy FF subjects were selected based on heathy criteria (McAllister et al., 2018), and low risk for CVD according to standards for American College of Sports Medicine (ACSM, 2013). From that total 9 were able to complete the study. Participants were excluded if they were current cigarette smokers, taking prescription drugs, and symptomatic for any cardiometabolic, neurological, or musculoskeletal disorders.

Study Design

The investigation used a double-blinded, cross-over design comparing curcumin supplementation and placebo were evaluated for OS using a dual stress challenge. Heat was used to evaluate cardiovascular responses to structural burn stress during a victim search and clear exercise protocol during three sessions (McAllister et al., 2018). Wearing fire protective equipment (PPE) the FFs consisting of two teams of 5 participated in following fire suppression exercises involving sessions 1) no heat (no available for NMR study), 2) heat + antioxidant (curcumin) and heat + placebo, and 3) was a repeated of session 2. Time between sessions 2 and 3 used a 7-day washout period to remove supplement treatment residue effect from the body. Supplementation during sessions 2 and 3 were randomly assigned. The curcumin (Curcufresh ™, Now Foods, Bloomingdale, IL) or placebo was given 45 min prior to the exercise as three capsules with 1.5 g each of curcumin/69 mg of curcuminoids. The exercise protocol for this study includes a 20 min victim search and clear of dummies weighing 90 to 100 kg located randomly throughout the tunnel as the FF were confronted by different obstacles (McAllister et al., 2018). A heated tunnel was used to simulate a structural fire that consisted of side-by-side heat houses connected to a 20 m long tunnel. The simulated heat consisted of fires maintained on multiple wood pallets burning inside, outside and on top of the heat-house. Temperatures in the 20 m tunnel heat-house inside floor and ceiling for no heat and first heat trial was 8.9 and 265.6 and 10.5 and 604.4 ℃, respectively. It is important to note that for each of the five member teams, two FF remained outside to extinguish the fire and three FF went into the heat house to complete each session. Details of victim search and clear exercise protocol and rates of curcumin supplementation during session 2 compared to the placebo treatment session 3 are reported in McAllister et al. (2018). Blood plasma samples collected in the previous study and used in

current investigation were drawn before exercise, immediately post-exercise and 30-minutes post exercise for testing sessions 2 and 3.

Blood Plasma Sampling (NMR)

Blood plasma samples were unavailable for testing from session 1 of the original study and could not be tested during NMR study. A total of 60 blood plasma samples were analyzed from 9 FF participants between the two sampling periods from the heat exercise sessions 2 and 3 for untargeted metabolome concentration comparisons. Furthermore, the blood plasma samples were compared between curcumin and placebo metabolite concentrations from the same protocol and sampling scheme. Specific detailed methodology conducted for ^{1}H NMR blood plasma preparation and buffer stock solution for sample preparation are discussed below. Spectral analysis was conducted using Chenomx NMR Suite 7.0 (Edmonton, Canada) to identify and quantify the metabolites associated with each blood plasma sample.

Blood Sampling and Procedures

Just prior to the exercise sessions and curcumin supplementation, blood samples were drawn from antecubital veins pre- and post-exercise, 30 min post exercise, and 24 h post exercise (4 blood plasma samples per session for each participant). The blood samples that were drawn from each FF were placed into a 12 ml EDTA anticoagulant sealed vacutainer tubes (McAllister et al., 2019). The tubes were centrifuged for 10 minutes at 2500 rpm at 4°C. Plasma was aliquoted and stored at -80°C for approximately 2 years prior to the current NMR study.

Plasma Preparation and Protein Removal

Samples of FF blood plasma was prepared using published methods protocol (Dona et al., 2014-S.3) and later with minor revision during filtration of blood plasma to remove excess

glycerol and proteins (Rivera-Santos et al., 2021). Specifically, plasma samples stored at -80°C were thawed to room temperature. It was noted in preliminary runs that large proteins prevented accurate identification of metabolites. To overcome this problem, 3 kDa molecular weight cutoff (MWCO) Omega membrane Microsep Advanced Centrifuge Tubes (Pall Corp., Ann Arbor, MI) were used to remove large proteins from the plasma. To remove glycerol, the tubes were rinsed in ultrapure water, filled to capacity, and centrifuged at 3,800 relative centrifugal field (RCF) for 10 min at 20° C. The water was removed from both the retentate vial and the collection tube of the ultrafiltration unit, and centrifugation was repeated. After two rounds of centrifugation, the unit was emptied and thoroughly dried. This process was found to reduce glycerol present in the tube filters to prevent contamination and artificially increase plasma sample concentrations. Immediately following glycerol removal, 900 µL of a bovine plasma sample (described below) was added to the concentrator and centrifuged at 3,800 RCF for 25 min at 20° C. The flow-through from the collection tube was stored into a vial at 4° C until ^{1}H NMR analysis (Rivera-Santos et al., 2021).

NMR Sample Preparation

Specific detailed of the methodology for conducting the NMR blood plasma preparation and analyses are detailed below. Blood plasma sample were aliquoted with 330 µL of an NMR referencing stock solution prepared as follows. The solution consisted of 200 mM sodium phosphate buffer, 1 mM 3-trimethylsilylpropionate 2, 2, 3, 3-d4 (TMSP-d4, Cambridge Isotope Labs DLM-48-5) and 0.1% (w/v) sodium azide (NaN_3) in 50% D2O. 50 mL of referencing solution was prepared consisting of 0.7356 g NaH_2PO_4 (sodium phosphate monobasic, anhydrous 99%), 0.5492 Na_2HPO_4 (disodium hydrogen phosphate, anhydrous 99%), 50 mg NaN3, and 8.613 mg of TMSP-d4 as an internal standard. Then 25 mL of ultrapure water was

added, vortexed, pH adjusted to 7.0 using concentrated HCl or 1 M NaOH. The buffer solution was then transferred to a 50 mL volumetric flask, and 25 ml of deuterium oxide (D2O, Cambridge Isotope Labs DLM-4-99) was added to the buffer solution, for a final volume of 50 mL which was thoroughly mixed. A 0.2 µM syringe filter was used to sterilize the referencing solution. The filtered plasma samples were mixed 1:1 with referencing solution and following mixing the entire 660 µL sample was transferred to clean NMR tubes (Wilmad LabGlass, 535-PP-7). The same solution was used for all samples described in this study to ensure uniformity during the experiment.

NMR Spectroscopy

Samples were analyzed with a Bruker Avance III-HD 500 MHz spectrometer equipped with a BBFO Probe (Bruker, MA, USA). Samples were run under automation mode using a SampleJet autosampler, with all samples refrigerated at 4 °C until just prior to analysis. Data was acquired using a perfect-echo WATERGATE sequence (PE-WATERGATE, parameter set ZGESGPPE) (Adam et al., 2012) for 128 scans with an inter-scan delay of one second and a per-scan acquisition time of 3s. Including 3D-shimming, the total time of acquisition for each sample was approximately 17 minutes. Raw spectra were imported into TopSpin 4.0.8 (Bruker Corporation, Bilerica MA), automatically phased, baseline corrected, and referenced to the TMSP at 0 ppm. Processed spectra were then imported into Chenomx NMR Suite (Chenomx, Edmonton, Canada) software to identify individual metabolites and relative concentrations using the 500 NMR reference library and the Human Metabolomic Database (HMDB.ca).

Statistical Analyses

The follow statistical analyses were used to compare metabolite diversity and concentrations. The initial univariate analysis data were subjected to analysis of variance (ANOVA) using a crossover design with SAS® PROC GLM (SAS/STAT ver. 9.4). Means were separated using Fisher's Least Significant Difference (LSD). The analysis compared the metabolites and their concentrations between treatment protocols for sessions 2 and 3. Statistical significance was set at $P \leq 0.05$ and ANOVA values are expressed as means ± SE (standard error). Because more than two variables were compared for this study analysis, fold change and volcano plots consisting of fold change and t-test comparisons could not be conducted.

For multivariate analyses, all metabolite concentrations were analyzed using MetaboAnalyst 4.0 platform (https://www.metaboanalyst.ca). Binned data was preprocessed applying normalization, data transformation and pareto scaled. Data was normalized by sum to minimize the differences in concentrations of the plasma samples.

Multivariate statistical analyses were conducted included principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA) (Westerhuis et al., 2010, Sun et al., 2014). Principal component analysis (PCA) was conducted to obtain a score plot that shows percent variation. Results were used to determine predefined groups and remove metabolite concentrations outliers. Scores are weighted averages of the original variables and weighted profiles. The partial least squares discriminant analysis (PLS-DA) method uses supervised multivariate regression to obtain linear combinations of original variables (X) that can predict associations of metabolites into groups (classes). These statistical methods define, or measure class discrimination based on

prediction accuracy and separation distance based on the ratio between and within the group sum of squares (B/W ratio).

Two key variables used to measure model predictive ability (goodness-of-fit) were cross-validation using Q^2 values and permutation rate (significant if $p \leq 0.05$). Specificity, sensitivity, and classification rates (percentage samples correctly classified) were obtained within a 1000-fold cross-validation for PLS-DA and OPLS-DA models. In those models the Q^2 parameter represents the predictive ability of the model supporting the authenticity of the results and permutation is used to validate and test the degree of over-fitting of the model (Sun et al., 2013). The OPLS-DA model maximized the covariance between measured data (X-variable data, concentrations from NMR spectra) and (Y-variable, predictive). Score plots combining the correlation from OPLS-DA models were used to distinguish differences in concentrations among groups. Cross-validation was used to verify if the model differentiated metabolite concentrations.

If the models were positively crossed-validated for PLS-DA and OPLS-DA, then the Variable Importance in Projection (VIP) analysis could be used for individual metabolite discussion. VIP is a weighted sum of squares associated with amount of Y-variation explained for each dimension. This is a weighted sum of PLS-regression for PLS-DA and OPLS-DA. Each metabolite (variable) with a VIP weight > 1.0 from the analyses was determined have most variation in concentrations (Cho et al., 2008). Using VIP greater than 1 is considered an acceptable cutoff threshold to define relevant variables (metabolite concentrations) using PLS regression or linear classification model (Akarachantachote et al., 2014). The PLS-DA and OPLS-DA are supervised models that are used to indicate variations in metabolite datasets to predict groups or clusters of metabolite concentrations treatment effects.

Results

Table 2

List of Firefighter Blood Plasma Metabolite Changes Associated with a Fire Stress Challenge and Curcumin Supplementation Prior to the Challenge Between Three Times and Supplementation

Metabolites	Pooled Fire Exercise and Supplementation Protocol Sessions 2 & 3								
	Pre-Heat Exercise Challenge	Immediate Post-Heat Exercise Challenge	30 min Post-Heat Exercise Challenge	LSD	P ≤ 0.05	Among All Sampling Periods Concentrations Means mM		LSD	P ≤ 0.05
	Concentrations Means mM					Curcumin	Placebo		
2-Hydroxyisovalerate	0.013[a]	0.011	0.012	0.007	0.674	0.012	0.013	0.005	0.659
2-Hydroxyvalerate	0.081	0.074	0.094	0.050	0.578	0.079	0.085	0.041	0.794
β-Hydroxybutyrate	0.095	0.042	0.049	0.110	0.570	0.069	0.055	0.089	0.658
4-Aminobutyrate	0.041	0.041	0.047	0.035	0.918	0.047	0.037	0.028	0.512
Acetate	0.042	0.037	0.037	0.018	0.777	0.034	0.044	0.015	0.175

Table 2 (continued)

Acetone	0.030	0.021	0.027	0.022	0.714	0.025	0.027	0.018	0.780
Alanine	0.429	0.463	0.426	0.071	0.691	0.442	0.464	0.058	0.583
Asparagine	0.065	0.049	0.048	0.029	0.333	0.050	0.059	0.024	0.365
Betaine	0.131	0.110	0.132	0.041	0.461	0.122	0.125	0.033	0.727
Carnitine	0.047	0.033	0.039	0.024	0.536	0.035	0.043	0.020	0.345
Creatine	0.025	0.018	0.024	0.016	0.608	0.027[b]	0.017	0.013	0.608
Creatinine	0.110	0.114	0.116	0.026	0.988	0.113	0.114	0.021	0.884
Formate	0.026	0.024	0.020	0.009	0.426	0.024	0.027	0.007	0.920
Glucose	3.53	4.03	3.78	1.001	0.517	4.087	3.445	0.815	0.517
Glutamate	0.078	0.059	0.046	0.055	0.538	0.062	0.061	0.045	0.988
Glutamine	0.499	0.481	0.484	0.063	0.835	0.491	0.485	0.052	0.970
Glycerol	0.183	0.173	0.193	0.456	0.654	0.186	0.181	0.371	0.822
Glycine	0.134	0.168	0.139	0.064	0.472	0.161	0.133	0.052	0.334
Histidine	0.067	0.052	0.045	0.031	0.229	0.049	0.062	0.052	0.229
Isoleucine	0.094	0.082	0.070	0.032	0.252	0.081	0.083	0.026	0.645

Table 2 (continued)

Lactate	**2.387B**	**4.830A**	**2.821AB**	**2.109**	**0.047**	3.732	3.044	1.717	0.388
Leucine	0.162	0.159	0.147	0.026	0.439	0.157	0.156	0.021	0.970
Lysine	0.138	0.099	0.139	0.086	0.490	0.112	0.138	0.069	0.466
Mannose	0.079	0.048	0.061	0.034	0.118	0.053	0.072	0.027	0.106
Methionine	0.028	0.017	0.017	0.012	0.111	0.020	0.022	0.010	0.458
Phenylalanine	0.027	0.019	0.018	0.016	0.321	0.019	0.025	0.013	0.315
Proline	0.163	0.141	0.148	0.056	0.731	0.152	0.149	0.046	0.871
Pyroglutamate	0.045	0.023	0.031	0.042	0.393	**0.016B**[b]	**0.052A**	**0.034**	**0.032**
Pyruvate	0.036	0.092	0.036	0.070	0.184	0.066	0.047	0.057	0.481
Threonine	0.031	0.017	0.029	0.034	0.672	0.021	0.030	0.028	0.419
Tyrosine	0.044	0.038	0.029	0.019	0.298	0.037	0.038	0.015	0.709
Urea	4.645	4.560	4.511	0.956	0.857	**4.168B**	**5.020A**	**0.778**	**0.033**
Valine	0.165	0.149	0.120	0.063	0.468	0.151	0.139	0.051	0.660
3-Methylhistidine	0.027	0.033	0.032	0.027	0.775	0.035	0.026	0.022	0.582

Table 2 (continued)

[a] Numbers represent mean metabolite concentrations mM. Mean concentration data followed by a different letter was significantly different ($p \leq 0.05$) using Fisher's LSD . [b] Refers to univariate analysis significant fold-change (FC > 1.5 difference) indicating up or downregulation for supplement comparisons.

A total of 34 metabolite were consistently identified from the blood plasma subsamples (Table 2). An additional 10 metabolites were irregularly detected and at low concentrations. These included 2-aminobutyrate, 2-hydroxybutyrate, 2-oxovalerate, arginine, choline, citrate, erythritol, ornithine, sarcosine, and serine.

Univariate Analyses

Analysis of variance (ANOVA) was conducted comparing individual metabolite concentration between pre-, immediately post-exercise, 30 min post exercise, and curcumin oral supplementation versus placebo and between times by supplements. There were no significant interactions between times x supplement and between the three sampling times for the 34 metabolites except lactate (Table 2). Lactate significantly upregulated ~2.0 mM/L immediately following exercise with heat but within 30 min post exercise concentrations returned to pre-exercise levels. When curcumin supplementation was compared to the placebo across blood sampling dates, urea and pyroglutamate were significantly lower following the curcumin supplementation. No other metabolites were significantly impacted using curcumin supplementation compared to the placebo during the study.

Univariate analysis further compared curcumin supplement to the placebo using paired FC analysis and volcano plots to highlight up or downregulated features for individual metabolites during the study. From the comparison, significant FC analysis listed three metabolites including creatine (1.67 -$\log^2$) upregulated while pyroglutamate (0.40) and pyruvate (0.66) were downregulated in the study. The remaining 31 metabolites fell below the threshold FC designated limit of 1.5 and were considered similar between the treatments. However, when volcano plot analysis was conducted none of the metabolite concentrations reach threshold or significance with t-test excluding the three from further consideration. The volcano plot analysis

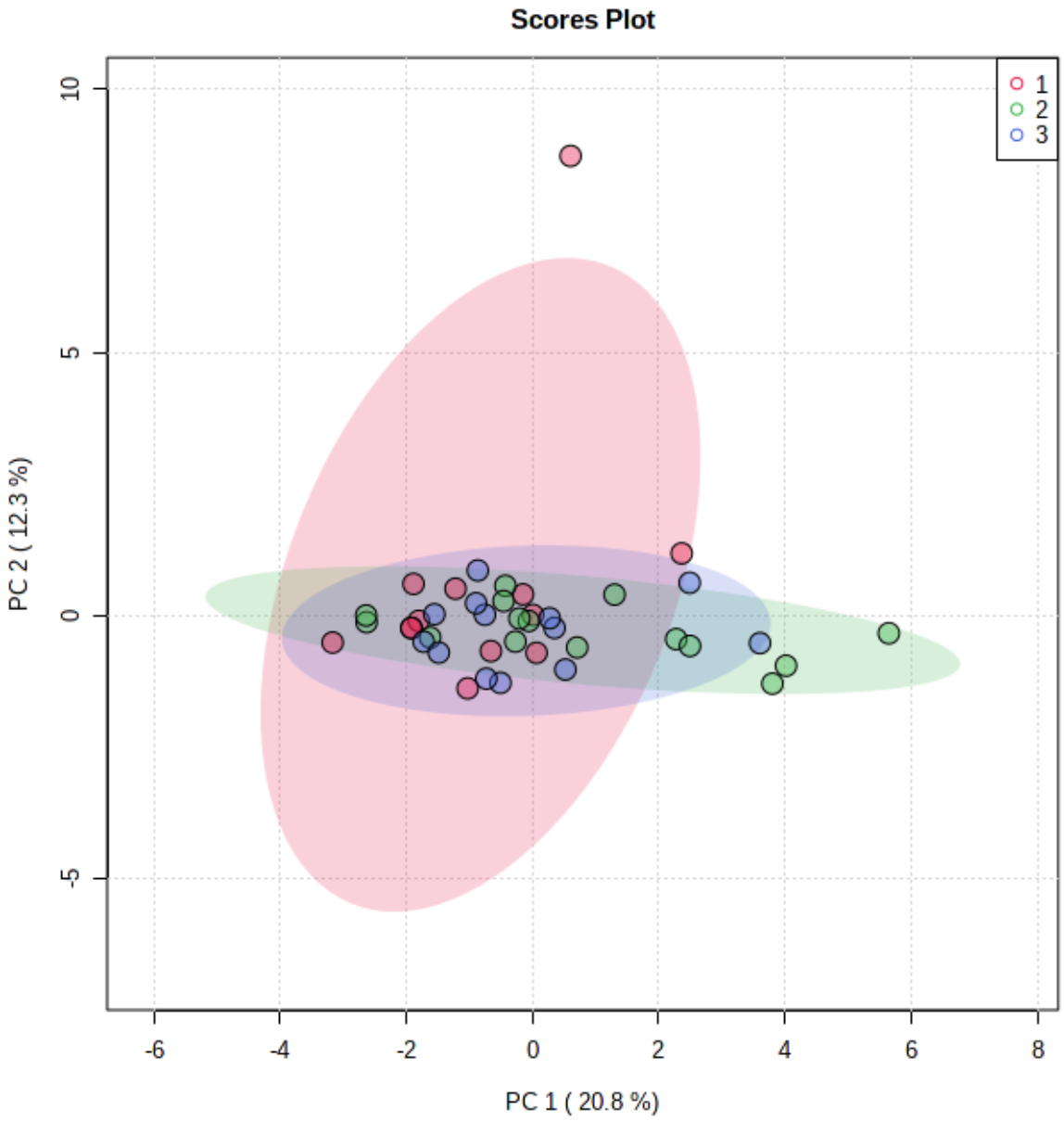
test is more stringent and supported the ANOVA analyses showing none of the three metabolites were significantly different between curcumin versus placebo. Lactate was similar when compared between curcumin versus placebo ingestions indicating that that the exercise with heat protocol was the likely cause of its transient upregulation of the metabolite immediately post-exercise and not supplementation.

Multivariate Analysis (Sampling Times)

Multivariate analysis was conducted comparing metabolite concentration data between the three sampling times pre-exercise, immediately following and 30 min post exercise. PCA analysis was used to visualize group separations for the metabolite concentrations between the three sampling times (Figure 3). Group separation of the metabolites visualized in the score plot shows a minimal trend towards select metabolite concentrations increasing immediately after exercise but downregulated to pre-exercise levels at 30 min post exercise. Total percent variations covered was 32.1% for components 1 and 2 and overall total of 55.6% for first five principal components is considered low. To confirm a potential trend immediately post-exercise, the metabolite concentrations were visualized using the models PLS-DA and OPLS-DA. However, cross-validation did not support the models PLS-DA using Q^2 coefficient (-0.35) and permutation value p = 0.90 (1000) (Figure A3). For OPLS-DA analysis the Q^2 coefficient (-0.86) and permutation value p = 1.0 (1000) did not support variations in metabolite concentrations that occurred following exercise (Figure A4). Since the models were not validated VIP scores for individual metabolites were not considered here. Results from the multivariate analyses overall did not support metabolite concentration differences between the treatment times of sampling or use of the supplementation.

Figure 3

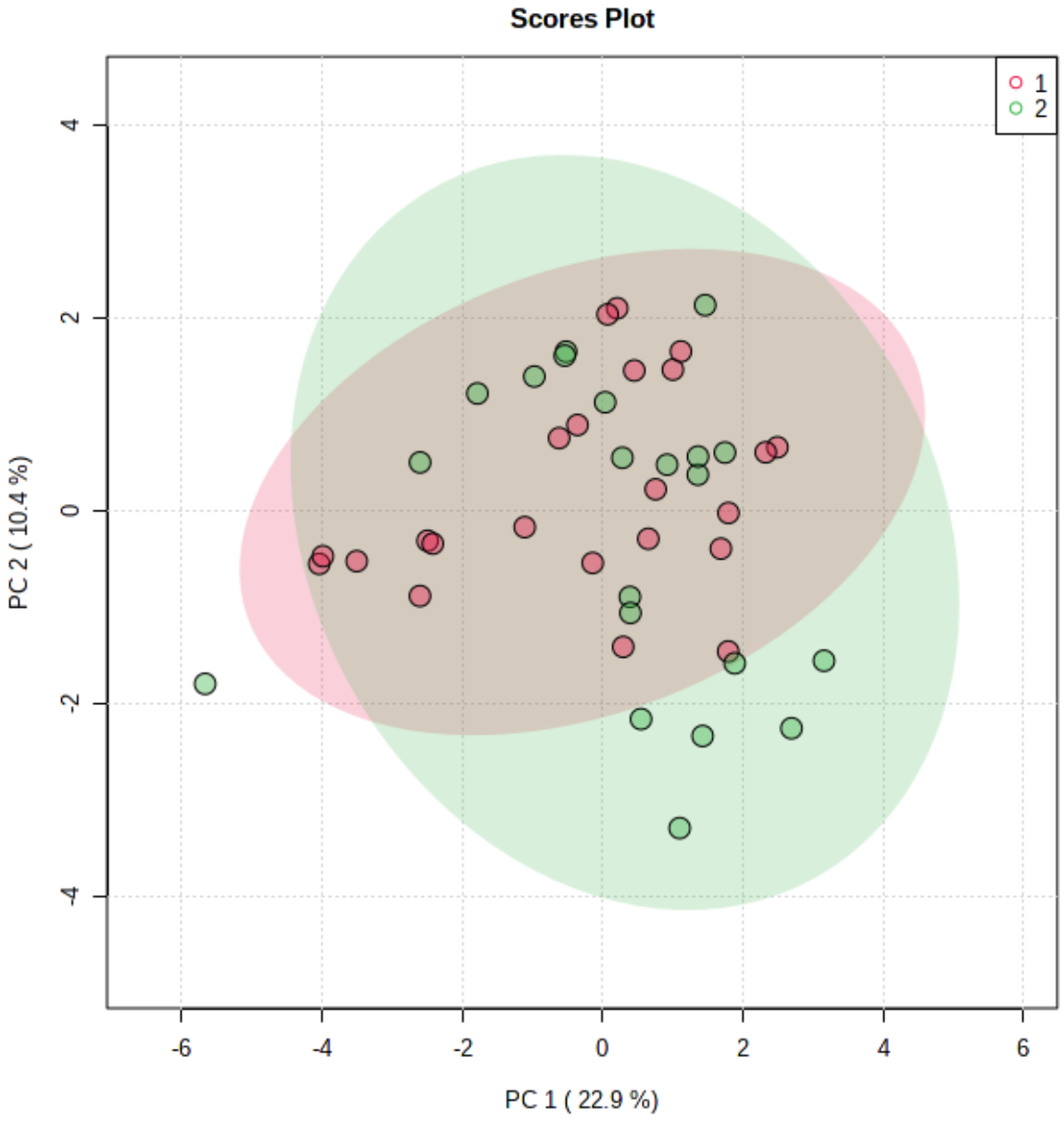
PCA Score Plot (Curcumin Sampling Times)



Note: Score plot of PCA model for principal components 1 and 2 explaining variance between three sampling periods pre-heat exercise (1), post-exercise (2) and 30 min after exercise (3).

Figure 4

PCA Score Plot (Curcumin Versus Placebo)



Note: Score plot of PCA model for principal components 1 and 2 explaining variance between metabolite concentrations between (1) curcumin and (2) placebo groups.

Multivariate Analysis (Curcumin versus Placebo)

PCA comparison of curcumin versus placebo concentrations determined that the first five principal components total variations were 54.5% (data not shown) while principal components 1 and 2 contained 33.3% of the variation (Figure 4). This total variation is low indicating that the PCA model marginally separated the curcumin versus placebo metabolite concentrations. For increased visualization of the supplement and placebo metabolite concentrations, PLS-DA and OPLS-DA models were used for comparison and for identifying important metabolites using VIP analysis within the treatments. Cross-validation values did not support the model's indicative of no treatment variations. Using the PLS-DA model, the Q^2 coefficient (-0.250) and permutation value p = 0.95 (1000) (Figure A5) and those for OPLS-DA model for Q^2 coefficient was -0.223 and permutation value at p = 0.85 (1000) (Figure A6) indicated no benefit from curcumin versus the placebo. The VIP scores for individual metabolites were not relevant since the models were not acceptable following cross-validation.

Discussion

A total of 34 metabolites and their concentrations were identified from the blood plasma samples (Table 2). An additional 10 metabolites could not be compared due to infrequent detection in the samples. Using ANOVA comparing the 34 metabolites, lactate was the only one that was significantly different between time periods pre- and immediately post-exercise, and at 30 min post exercise. Concentrations of lactate were significantly upregulated immediately following exercise with heat but returned to pre-exercise levels at 30 min post exercise. Curcumin supplementation and the placebo for lactate concentrations were similar. This indicates that the physical exercise protocol with heat might be the primary reason for the increase in lactate. In another study, using a handgrip protocol, venous blood plasma lactate was

shown to increase significantly immediately post-exercise from 2.9 to 11.0 mM/L (Dube et al., 2005). Normal lactate levels in blood plasma can range from 0.6 to 3.6 mM/L (Hashim et al., 2018) while levels can increase following sampling over time and in storage (Schroeder & Hansen, 2005). Transient lactate increases following exercise supported a ROS signaling response that caused antioxidant promoting OS defenses thereby offsetting the negative effects of the transient OS (Tauggenberger et al., 2019). The increase in lactate following the FF exercise protocol in the current study may be partially responsible for the reduced OS reported in the initial study (McAllister et al., 2018). Concerning cardiovascular disease, lactate is reported to be higher in individuals with insulin resistance and adiposity thus leading to hypertension (Digirolamo et al., 1992). However, the mean levels of lactate using ^{1}H NMR in this study were never outside their normal range for the FF. Lactate is formed from pyruvate primarily in muscle and red blood cells during intense anerobic activities as muscle respiration is reduced (Brooks, 1986). Lactate dehydrogenase catalytically activates the reaction to form lactate from pyruvate when triggered with lower oxygen levels (Schumann et al., 2002) The transient increase in lactate by FF in the current study was the direct result of the catalytic activity.

Analysis showed that glucose levels were lower than normal in the blood plasma using ^{1}H NMR but were similar pre-, immediately post-exercise and 30 min post exercise. One study reported that storage of blood plasma at 1° to 6° C for 35 to 42 days resulted in lower blood glucose readings than immediately after sampling (Adams et al., 2015). In addition, glycerol levels, often an indicator of adipose triglyceride lipolysis, rarely exceeds 0.43 mM/L during increased fat metabolism but can increase to 1.6 saturation point in plasma and in urine (Nelson et al., 2011). The high levels of glycerol of the current study may be partially due to a glycerol residue associated with the filter tubes (see methods) used to removal large proteins that

interfered with spectral signature data during preliminary studies using ^{1}H NMR. Urea levels were significantly lower following curcumin supplementation than placebo while no differences occurred between pre- and immediately post-exercise protocol in the NMR study. Contrary to these findings the amino acids arginine and ornithine associated with the urea metabolic pathway were low when present and inconsistently identified in the samples. A study reported that urea can provide cardiovascular protection as an antioxidant (Wang et al., 1999). It was suggested that urea repressed NO formation from cardiac tissues that supported reoxygenation injury (Metheis et al., 1992). Contrary to those studies, urea increases transcriptional factor Gadd153/CHOP associated with greater OS in the kidney medulla and ROS levels with chronic renal failure in mice (Zhang et al., 1999; D'Apolito et al., 2010). In this study there is no evidence that increased urea formation supported reduced OS.

Pyroglutamate, an intermediate in the formation of glutamate from glutamine or the reverse, was significantly lower following curcumin supplementation. There were no observable trends between glutamine and glutamate as all concentrations were similar between pre-, immediately post-exercise, and at 30 min post exercise (Table 2). It was previously reported that glutamine and curcumin reduced oxidative tissue damage and apoptosis compared to a control group in rats (Karatepe et al., 2010). However, no direct use of curcumin was correlated with increase glutamine to glutamate ratios except that pyroglutamate was reported to be in equilibrium with glutamate during forward or reverse reactions (Mena et al., 2005). Furthermore, uniformly conducted research understanding the role of curcumin in glutamate formation needs further investigation with the objective to reduce OS.

Multivariate analyses models PCA, PLS-DA and OPLS-DA did not support separation of the metabolite concentrations between sampling times and curcumin versus placebo using the

unsupervised PCA model or the two supervised models. Since neither of the supervised models were supported by cross-over validation, individual metabolite VIP scores were not relevant and could not be discussed.

In conclusion, lactate was the only metabolite that upregulated following exercise suggesting possible transient OS suppression that supported the results in the original study by McAllister et al. (2019). Comparing metabolite concentrations across sampling times, lactate increased as expected post-exercise suggesting metabolic signaling activity leading to antioxidant formation against transient OS from exercise with heat protocol. Multivariate analyses during the study did not support the PCA, PLS-DA and OPLS-DA models in this study suggesting that no visible concentration differences occurred and that other data types may be more appropriate for these multivariate models.

CHAPTER V

^{1}H NMR ANALYSIS OF KETONE SALT SUPPLEMENTATION ON OXIDATIVE STRESS BIOMARKERS IN FIREFIGHTERS BLOOD PLASMA FOLLOWING SIMULATED EXERCISE PROTOCOL

Abstract

Previously a 7-day ketone salt oral supplementation was evaluated to determine its impacts on transient oxidative stress (OS) following a physical exercise protocol using firefighters (FF) in personal protection equipment (PPE). Blood plasma samples were obtained to compare changes in specific antioxidant or OS biomarkers. Results suggested that 7-day supplementation of ketone salt caused no biomarker treatment or treatment by time effects for OS levels. In the present study, two-year-old blood plasma subsamples from that investigation were reevaluated using a ^{1}H Nuclear Magnetic Resonance (NMR) platform. The purpose was to identify untargeted metabolites and their concentrations to further define benefits of ketone salt supplementation on metabolic changes associated with OS. A total of 38 metabolites were identified while an additional 8 metabolites were inconsistently detected. Mean separation using analysis of variance (ANOVA) Fisher's LSD at ($p \leq 0.05$) was conducted to compare plasma samples at pre- and post-exercise, 30 min and at 24 h post exercise. Across those times, 13 metabolites significantly different concentrations. Lactate which increased post-exercise and at 30 min post exercise returned to pre-exercise levels at 24 h post exercise. The ketone bodies acetone and β-hydroxybutyrate increased post-exercise while branch chain amino acids (BCAA)

isoleucine and valine concentrations significantly decreased following exercise while leucine increased across the sampling times. The ketone salt supplementation resulted in significant upregulation of β-hydroxybutyrate, acetone, isoleucine, and leucine. Whereas the amino acids asparagine and valine downregulated compared to their placebo values. When time by supplementation was compared for the 38 metabolites, 4-aminobutyrate, acetone, carnitine, glutamate, and isoleucine had significant interactions between the two variables. Data was further analyzed by principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and orthogonal partial least-squares discriminant analysis (OPLS-DA). The PCA comparison moderately discriminated the ketone salts supplementation from placebo metabolites but could not discriminate them between the four blood plasma sampling times. Furthermore, PLS-DA and OPLS-DA models could not differentiate the metabolite concentrations pre-, post-exercise, 30 min, and 24 h post exercise or between ketone salt versus placebo. In conclusion, β-hydroxybutyrate, the three BCAAs and lactate concentrations were affected following the exercise protocol and/or ketone salt supplementation which may support. some level of reduced transient OS, but this could not be confirmed during this study. Furthermore, the metabolites detected from the stored blood plasma samples using ^{1}H NMR did not identify direct biomarkers for reducing OS supporting FF cardiovascular health.

Introduction

Firefighters (FF) are reported to have high levels of cardiovascular disease (CVD). When a death occurred on the job, CVD was directly or indirectly responsible for over of 45% line-of-duty fatalities (Smith, 2011; Sorteriades et al., 2011). General poor eating habits and low exercise levels of veteran FF contributed to various levels of metabolic syndrome. Factors contributing to the syndrome include high triglyceride levels, hypertension, hyperglycemia,

insulin resistance, high levels of low-density lipoproteins (LDL), and visceral obesity (Alberti et al., 2009; Alsherhri, 2010). These health issues are correlated with even greater increases in oxidant to antioxidant ratios. FF must wear personal protection equipment (PPE) which increases heat stress, leading to higher body temperatures supporting physical exhaustion that contribute to transient and chronic oxidative stress (OS) (Park et al., 2016). The chronic OS initiates persistent inflammation coupled with FF occupation duties is correlated with advanced arthrosclerosis that can ultimately lead to metabolic syndrome events during periods of intense on-the-job activities (Huang et al., 2013).

Dietary interventions and regular aerobic exercise have been used to reduce OS and associated metabolic markers in FF (Park et al., 2016; McAllister et al., 2018, 2019). Ketone diets or oral ketone salt supplementations were reported to reduce OS at the mitochondrial level and improve antioxidant capacity (McAllister et al., 2019). A review paper examined 16 studies on the impact of ingesting ketone and exercise performance (lower-body and endurance power) (Margolis & O'Fallon, 2020), overall, it was reported that there are inconsistent differences between exercise performances with ketone esters supplementation (acetone and β-hydroxybutyrate) or ingesting ketone salts. Metabolomic studies conducted using GC-MS and LC-MS platforms, reported that physical exercise with a green tea supplementation acutely increased β-hydroxybutyrate, which is implicated in reducing OS and other acute effects to metabolite diversity pre- or post-exercise (Jacobs et al., 2014). β-hydroxybutyrate is a signaling metabolite affecting different functions such as lipid metabolism, gene expression and metabolic rate that can impact OS (Newman & Verdin, 2017). In addition, the metabolite acting as a signaling molecule was reported to regulate neuron responses in sympathetic nervous system for regulating blood pressure and heart rate (Rezq & Abel-Rahman, 2016). Use of exogenous

supplementation of β-hydroxybutyrate can increase cellular protection (Cavaleri & Bashar, 2018). It is reported to be a histone deacetylase inhibitor which results in increased acetylation of promoter regions of genes responsible for transcription of antioxidants such as superoxide dismutase, catalase, and metallothionein supporting homeostasis with ROS (Shimazu et al., 2013). It is possible that increased metabolomic signals such as the β-hydroxybutyrate response reported in these studies may support some level of ROS protection.

With new platforms available to analyze biological fluids, including ^{1}H-NMR, large sets of metabolomic data can be generated to provide more comprehensive and detailed understanding of molecular processes than ever. This technology can provide more comprehensive investigations into important targeted or untargeted metabolites/concentrations that could not be determined from single samples with more traditional approaches. For example, the metabolomes of 80 professional football players were evaluated for heart rate, ratings of perceived exertion, and measures of physical work and performance from urinary metabolic profiles (Quintas et al., 2020). Results from this study showed significant associations between urine metabolic profiles and internal alterations of biochemical pathways and training adaptations. The metabolic analysis identified upregulated pathways including metabolites associated with physical work and performance including steroid hormone biosynthesis intermediates, and the phenylalanine pathway metabolism intermediates tryptophan. Using ^{1}H-NMR, Meisser et al. (2017) determined that plasma levels of glucose, glutamine, alanine, and branched-chain amino acids (BCAAs) decreased after prolonged exercise at higher altitude while levels of lipid biomarkers were similar at sea level, suggesting the involvement of several protein pathways. Similarly, a cycling time trial study elicited a 3.1-fold increase in the lipid metabolites 13-HODE + 9-HODE and cytokines (IL-6, IL-8, IL-10, TNF-α) responsible for OS and

inflammation, were identified from blood plasma using a metabolomic approach (Neiman et al., 2014). Metabolomic data are now available with NMR analytic platforms and are fast and reliable tools for understanding the functional impacts from dietary supplementation or exercise to monitor OS.

McAllister et al. (2019) evaluated blood plasma samples and compared OS specific targeted biomarkers concentrations following ketone salt supplement versus placebo and between pre- and post-exercise, 30 minutes and 24 hours post exercise. In that study, no treatment or treatment by time interaction effect was found for the targeted metabolic biomarkers including red blood cells, glutathione, oxidized glutathione, superoxide dismutase, catalase, total antioxidant capacity, malondialdehyde, and glucose. There was observed exercise induced increases in OS biomarker levels of SOD from pre- to post-exercise, and decreased levels of glutathione from pre- to 30 min post exercise. Ketone salt supplement contributed to significant increased ketone levels in the study but there were no indicated benefits for OS following exercise. However, this study only examined select targeted metabolites, and other untargeted metabolites could provide increased resolution, stand alone, or supportive data using the ^{1}H NMR platform. The stored blood plasma subsamples from the McAllister et al. (2019) ketone salt study provided a unique opportunity to increase the level of metabolic knowledge from the previous investigation attempting to reduce OS and improve cardiovascular health in FF (McAllister et al., 2019).

Objectives: The aim of this study was to determine physiological responses on stress from exercise, and ketone salt supplementation by monitoring changes in concentrations of untargeted metabolites using ^{1}H-NMR direct profiling of blood plasma collected from a previous

FF study (McAllister et al. 2019), and to correlate specific up or downregulation of metabolites and their associated pathways to infer OS associated cardiovascular health.

Methods

Participants

All participating FF met five participation criteria including: 1) be an active firefighter, 2) meet the American College of Sports Medicine (ACSM) low-risk guidelines (American College of Sports Medicine, 2013), 3) obtain 150 minutes of moderate-intensity aerobic activity or 60 minutes of vigorous intensity aerobic activity per week, 4) avoids select dietary supplements containing antioxidants or ergogenic aids, and 5) refrain from alcohol and caffeine prior to the experiment, Mississippi State University's Institutional Review Board (IRB) approved the study plan with those criteria. Other specific eligibility guidelines included that each subject be cleared for duty by a physician, followed by a general health screening, dietary history and physical activity readiness questionnaires and medical history report. During the initial screening participants had their body mass and height measured in addition to a body composition assessment for fat and lean muscle mass using seven-site skinfold procedure. Following these assessments blood was drawn from each participant to evaluated baseline metabolic levels. Following the selection process 9 of 10 participants were chosen to complete the 3-session experiment. The specific blood plasma samples obtained from the 9 FF during the past investigation by McAllister et al. (2019) and analyzed using ^{1}H NMR in current investigation cannot be tied to specific individuals.

Study Design

The investigation used a randomized double-blinded, cross-over design comparing ketone salts mixture containing β-OHB supplementation and placebo control as reported previous (McAllister, 2019). Three sessions were conducted with blood samples taken during the last two sessions of the study. The sessions included the following:

Session 1:

Baseline anthropometric measures were obtained including height, body mass, and mass with PPE. Without PPE gear, a graded exercise treadmill test (using a Woodway Treadmill Waukesha, WI)) was used with increasing speed and incline every 3 min to obtain VO_{2peak} (McAllister et al., 2019). The VO_{2peak} test was performed to exhaustion while wearing comfortable clothing.

Following session 1, the participants started a 7-day supplementation with either exogenous ketone salt or a calorie flavoring containing placebo twice per day. Specifically, half the participants ingested ketone salt (KS) contained β-hydroxybutyrate (β-OHB) (Keto//OS® MAXTM, Melissa TX) and half the placebo (PLA) control consisting of maltodextrin that was colored and flavored (McAllister et al., 2019). The KS contained 7 g of KS, erythritol, L-tuarine, fermented L-leucine, citric acid, natural flavoring, vegetable juice color, stevia, xanthan gum, beta carotene, and 920 mg of sodium. A washout period of 7-days was used to eliminate all traces of the supplement after Session 1.

Session 2

Following the washout period, the participants returned to the laboratory. For session 2, with randomization resulting in half the participants ingesting KS and half PLA following at

least an 8-hour fast. Participants ingested the supplements 35 min before the physical stress task 60% VO_2peak and 2% grade with PPE attire that was conducted for 35 min. The PPE included turnout gear, self-contained breathing apparatus, air tank, and gloves. Blood plasma samples were drawn pre- and post-exercise, 30 min and 24 h post exercise. Following session 2, a 7-day supplement washout period occurred prior to starting of session 3.

Session 3

This session was conducted on day 8 following the washout period with participants supplementation were alternated from those FF given during session 2. KA and PLA supplementation type and physical exercise protocol were same as in session 2. Supplementation was given 35 min prior to the exercise that was conducted for a 35 min period. Blood plasma samples were obtained using the same methods as in Session 2.

Blood Sampling and Procedures

Just prior to the exercise, blood samples were drawn from antecubital veins pre- and post-exercise, 30 min post exercise, and 24 h post exercise (4 blood plasma samples per session for each participant) of the exercise protocol during those sessions. The blood samples were placed into a 12 ml EDTA anticoagulant sealed vacutainer tubes (McAllister et al., 2019). The tubes were centrifuged for 10 minutes at 2500 rpm at 4°C. Plasma was aliquoted and stored at -80°C for approximately 2 years prior to the current NMR study.

Plasma Preparation and Protein Removal

Samples of FF blood plasma was prepared using published methods protocol (Dona et al., 2014-S.3) and later with minor revision during filtration of blood plasma to remove excess glycerol and proteins (Rivera-Santos et al., 2021). Specifically, plasma samples stored at -80°C

were thawed to room temperature. It was noted in preliminary runs that large proteins prevented accurate identification of metabolites. To overcome this problem, 3 kDa molecular weight cutoff (MWCO) Omega membrane Microsep Advanced Centrifuge Tubes (Pall Corp., Ann Arbor, MI) were used to remove large proteins from the plasma. To remove glycerol, the tubes were rinsed in ultrapure water, filled to capacity, and centrifuged at 3,800 relative centrifugal field (RCF) for 10 min at 20° C. The water was removed from both the retentate vial and the collection tube of the ultrafiltration unit, and centrifugation was repeated. After two rounds of centrifugation, the unit was emptied and thoroughly dried. This process was found to reduce glycerol present in the tube filters to prevent contamination and artificially increase plasma sample concentrations. Immediately following glycerol removal, 900 µL of a bovine plasma sample (described below) was added to the concentrator and centrifuged at 3,800 RCF for 25 min at 20° C. The flow-through from the collection tube was stored into a vial at 4° C until ^{1}H NMR analysis (Rivera-Santos et al., 2021).

NMR Sample Preparation

A total of 72 blood plasma samples were analyzed using ^{1}H NMR spectrometry from the 9 FF participants comparing metabolites and concentrations associated with the ketone supplementation and exercise sessions conducted during sessions 2 and 3. Specific detailed of the methodology for conducting the NMR blood plasma preparation and analyses are detailed below.

Blood plasma samples were aliquoted with 330 µL of an NMR referencing stock solution prepared as follows. The solution consisted of 200 mM sodium phosphate buffer, 1 mM 3-trimethylsilylpropionate 2, 2, 3, 3-d4 (TMSP-d4, Cambridge Isotope Labs DLM-48-5) and 0.1% (w/v) sodium azide (NaN_3) in 50% D_2O. 50 mL of referencing solution used included 0.7356 g

NaH_2PO_4 (sodium phosphate monobasic, anhydrous 99%), 0.5492 Na_2HPO_4 (disodium hydrogen phosphate, anhydrous 99%), 50 mg NaN_3, and 8.613 mg of TMSP-d4 as an internal standard. Then 25 mL of ultrapure water was added, vortexed, pH adjusted to 7.0 using concentrated HCl or 1 M NaOH. The buffer solution was then transferred to a 50 mL volumetric flask, and 25 ml of deuterium oxide (D_2O, Cambridge Isotope Labs DLM-4-99) was added to the buffer solution, for a final volume of 50 mL which was thoroughly mixed. A 0.2 µM syringe filter was used to sterilize the referencing solution. The filtered plasma samples were mixed 1:1 with referencing solution and following mixing the entire 660 µL sample was transferred to clean NMR tubes (Wilmad LabGlass, 535-PP-7). The same solution was used for all samples described in this study to ensure uniformity during the experiment.

NMR Spectroscopy

The 72 samples were analyzed with a Bruker Avance III-HD 500 MHz spectrometer equipped with a BBFO Probe (Bruker, MA, USA). Samples were run under automation mode using a SampleJet autosampler, with all samples refrigerated at 4 °C until just prior to analysis. Data was acquired using a perfect-echo WATERGATE sequence (PE-WATERGATE, parameter set ZGESGPPE; Adam et al., 2012) for 128 scans with an inter-scan delay of one second and a per-scan acquisition time of 3s. Including 3D-shimming, the total time of acquisition for each sample was approximately 17 minutes. Raw spectra were imported into TopSpin 4.0.8 (Bruker Corporation, Bilerica MA), automatically phased, baseline corrected, and referenced to the TMSP at 0 ppm. Processed spectra were then imported into Chenomx NMR Suite (Chenomx, Edmonton, Canada) software to identify individual metabolites and relative concentrations using the 500 NMR reference library and the Human Metabolomic Database (HMDB.ca).

Statistical Analyses

The follow statistical analyses were used to compare metabolite diversity and concentrations. Initial univariate analysis data were subjected to analysis of variance (ANOVA) using a crossover design with SAS® PROC GLM (SAS/STAT ver. 9.4). Means were separated using Fisher's Least Significant Difference (LSD). The analysis compared the metabolites and their concentrations between sessions 2 and 3. Statistical significance was set at $P \leq 0.05$ and ANOVA values are expressed as means ± SE (standard error). Because more than two variables were compared for this study analysis, fold change and volcano plots consisting of fold change and t-test comparisons could not be conducted.

For multivariate analyses, all metabolite concentrations were analyzed using MetaboAnalyst 4.0 platform (https://www.metaboanalyst.ca). Binned data was preprocessed applying normalization, data transformation and pareto scaled. Data was normalized by sum to minimize the differences in concentrations of the plasma samples.

For visualization of metabolite concentration data between the treatments, multivariate statistical analyses were conducted included principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA) (Westerhuis et al., 2010, Sun et al., 2014). Principal component analysis (PCA) was conducted to obtain a score plot that shows percent variation. Results were used to determine predefined groups and remove metabolite concentrations outliers. Scores are weighted averages of the original variables and weighted profiles. The partial least squares discriminant analysis (PLS-DA) method uses supervised multivariate regression to obtain linear combinations of original variables (X) that can predict associations of metabolites into groups (classes). These

statistical methods define, or measure class discrimination based on prediction accuracy and separation distance based on the ratio between and within the group sum of squares (B/W ratio).

Two key variables used to measure model predictive ability (goodness-of-fit) were cross-validation using Q^2 values and permutation rate (significant if $p \leq 0.05$). Specificity, sensitivity, and classification rates (percentage samples correctly classified) were obtained within a 1000-fold cross-validation for PLS-DA and OPLS-DA models. In those models the Q^2 parameter represents the predictive ability of the model supporting the authenticity of the results and permutation is used to validate and test the degree of over-fitting of the model (Sun et al., 2013). The OPLS-DA model maximized the covariance between measured data (X-variable data, concentrations from NMR spectra) and (Y-variable, predictive). Score plots combining the correlation from OPLS-DA models were used to distinguish differences in concentrations among groups. Cross-validation was used to verify if the model differentiated metabolite concentrations.

If the models were positively crossed-validated for PLS-DA and OPLS-DA, then the Variable Importance in Projection (VIP) analysis could be used for individual metabolite discussion. VIP is a weighted sum of squares associated with amount of Y-variation explained for each dimension. This is a weighted sum of PLS-regression for PLS-DA and OPLS-DA. Each metabolite (variable) with a VIP weight > 1.0 from the analyses was determined have most variation in concentrations (Cho et al., 2008). Using VIP greater than 1 is considered an acceptable cutoff threshold to define relevant variables (metabolite concentrations) using PLS regression or linear classification model (Akarachantachote et al., 2014). The PLS-DA and OPLS-DA are supervised models that are used to indicate variations in metabolite datasets to predict groups or clusters of metabolite concentrations treatment effects.

Results

A total of 38 metabolites were identified consistently from the blood plasma samples (Table 3). An additional 8 metabolites were detected at low levels or infrequently including 2-aminobutyrate, 2-oxovalerate, arginine, choline, citrate, ornithine, sarcosine, and uridine.

Univariate Analyses

Data from the study were compared only between the two blood sampling sessions 2 and 3 since there were no available samples remaining from session 1. Using ANOVA, five metabolites had time x supplement significant interactions including 4-aminobutyrate, acetone, carnitine, glutamate, and isoleucine. When metabolite concentrations were compared between times of sampling, 13 metabolites had significantly different concentrations between the four sampling periods. Of those, acetones and β-hydroxybutyrate increased immediately after exercise and returning to pre-exercise levels within 24 h post exercise. Furthermore, the two ketone bodies were significantly upregulated compared to the placebo following ketone salt supplementation with exercise times of blood sampling. Lactate was significant upregulated immediately after exercise but decreased at 30 min post exercise and returned to pre-exercise levels within 24 h post exercise. The three BCAAs leucine, isoleucine and valine significantly differed in concentrations following exercise. Isoleucine and valine were downregulated post-exercise while both returned to pre-exercise levels at 24 h post exercise. Leucine upregulation occurred post-exercise but returned to pre-exercise levels when measured at 30 min and 24 h post exercise. All 13 metabolites that significantly differed across sampling times returned to pre-exercise levels at 24 h post exercise. An exception was phenylalanine, the concentration levels downregulate at 30 min post exercise later and 24 h post exercise.

Table 3

Firefighter Blood Plasma Metabolite Changes Associated with a Fire Stress Exercise Challenge and Ketone Salt Supplementation

Metabolites	Pooled Fire Exercise and Supplementation Protocol Sessions 2 & 3									
	Blood Plasma Sampling Times During Each Session									
	Pre-Exercise	Post Exercise	30 min Exercise	24 h Exercise	LSD	P≤ 0.05	Among Sampling Periods Concentrations Means mM/L		LSD	P≤ 0.05
	Concentrations Means mM/L						Ketone	Placebo		
2-Hydroxyisovalerate	0.012[a]	0.014	0.013	0.012	0.003	0.880	0.012	0.014	0.006	0.114
2-Hydroxyvalerate	0.074	0.063	0.055	0.083	0.027	0.070	0.062	0.074	0.032	0.231
β-Hydroxybutyrate	**0.034AB[b]**	**0.054A**	**0.032AB**	**0.018B**	**0.034**	**0.032**	**0.049A**	**0.020B**	**0.038**	**0.017**
4-Aminobutyrate*[c]	**0.014B**	**0.041A**	**0.026AB**	**0.021AB**	**0.021**	**0.001**	0.017	0.033	0.021	0.120
Acetate	**0.048B**	**0.067A**	**0.050B**	**0.045B**	**0.016**	**0.050**	0.053	0.051	0.022	0.962

Table 3 (continued)

Acetone*	**0.013C**	**0.034A**	**0.026B**	**0.012C**	**0.006**	**0.001**	**0.031A**	**0.013B**	**0.007**	**0.001**
Alanine	0.433	0.496	0.444	0.458	0.060	0.119	0.436	0.478	0.067	0.063
Asparagine	0.084	0.073	0.070	0.082	0.060	0.545	**0.063B**	**0.090A**	**0.020**	**0.007**
Betaine	**0.124BC**	**0.176A**	**0.146B**	**0.117C**	**0.027**	**0.001**	0.149	0.134	0.033	0.309
Carnitine	0.062	0.072	0.057	0.046	0.038	0.114	0.055	0.063	0.015	0.287
Creatine	0.025	0.023	0.023	0.020	0.009	0.619	0.028	0.018	0.017	0.330
Creatinine	0.097	0.106	0.100	0.098	0.016	0.711	0.094	0.106	0.019	0.187
Erythritol	**0.136BC**	**0.837A**	**0.405B**	**0.050C**	**0.277**	**0.001**	0.370	0.361	0.269	0.672
Formate	0.037	0.040	0.035	0.032	0.006	0.072	0.037	0.036	0.008	0.937
Glucose	3.938	3.814	3.357	3.965	0.695	0.192	3.994	3.55	1.027	0.096
Glutamate	0.062	0.048	0.051	0.062	0.052	0.877	0.059	0.052	0.049	0.913
Glutamine	0.497	0.506	0.466	0.520	0.061	0.392	0.499	0.495	0.050	0.767
Glycerol	**1.531B**	**1.892A**	**1.679AB**	**1.402B**	**0.315**	**0.004**	1.493	1.760	0.468	0.271
Glycine	0.085	0.083	0.034	0.097	0.059	0.194	0.070	0.079	0.044	0.650
Histidine	**0.067A**	**0.059AB**	**0.036B**	**0.081A**	**0.024**	**0.003**	0.056	0.064	0.027	0.402

Table 3 (continued)

Isoleucine*	**0.068AB**	**0.043C**	**0.049BC**	**0.076A**	**0.020**	**0.018**	**0.033B**	**0.082A**	**0.018**	**0.001**
Lactate	**1.220C**	**1.997A**	**1.561B**	**1.220C**	**0.298**	**0.001**	1.490	1.524	0.318	0.767
Leucine	**0.178B**	**0.201A**	**0.172B**	**0.162B**	**0.023**	**0.038**	**0.198A**	**0.160B**	**0.022**	**0.001**
Lysine	0.157	0.213	0.166	0.195	0.056	0.071	0.187	0.178	0.071	0.766
Mannose	0.063	0.060	0.063	0.053	0.029	0.977	0.055	0.064	0.030	0.521
Methionine	0.025	0.026	0.025	0.027	0.008	0.775	0.023	0.028	0.008	0106
N,N-Dimethylglycine	0.030	0.029	0.028	0.028	0.008	0.955	0.003	0.003	0.001	0.070
Ornithine	0.012	0.014	0.008	0.013	0.028	0.968	0.007	0.017	0.015	0.414
Phenylalanine	**0.026A**	**0.018AB**	**0.007B**	**0.012B**	**0.013**	**0.043**	0.012	0.019	0.011	0.206
Proline	0.179	0.176	0.159	0.215	0.041	0.175	0.187	0.177	0.036	0.682
Pyroglutamate	0.025	0.028	0.033	0.018	0.024	0.789	0.030	0.022	0.023	0.260
Pyruvate	0.032	0.030	0.020	0.022	0.021	0.693	0.022	0.029	0.013	0.490
Serine	0.052	0.046	0.047	0.039	0.065	0.931	0.031	0.055	0.058	0.406
Threonine	0.026	0.036	0.033	0.049	0.040	0.725	0.041	0.031	0.030	0.387
Tyrosine	0.044	0.042	0.041	0.055	0.041	0.155	**0.036B**	**0.054A**	**0.013**	**0.008**

Table 3 (continued)

Urea	4.333	4.480	4.153	4.651	0.632	0.455	4.460	4.346	0.799	0.883
Valine	**0.179AB**	**0.132BC**	**0.092C**	**0.196A**	**0.054**	**0.003**	**0.105B**	**0.191A**	**0.042**	**0.002**
3-Methylhistidine	0.029	0.017	0.027	0.016	0.021	0.422	0.024	0.021	0.020	0.684

[a] Numbers represent mean metabolite concentrations mM. [b]Mean concentration data followed by a different letter was significantly different ($p \leq 0.05$) using Fisher's LSD. [c] *Refers to univariate analysis significant fold change (FC > 1.5 difference) indicating up or downregulation of a metabolites as compared between supplement and placebo comparisons.

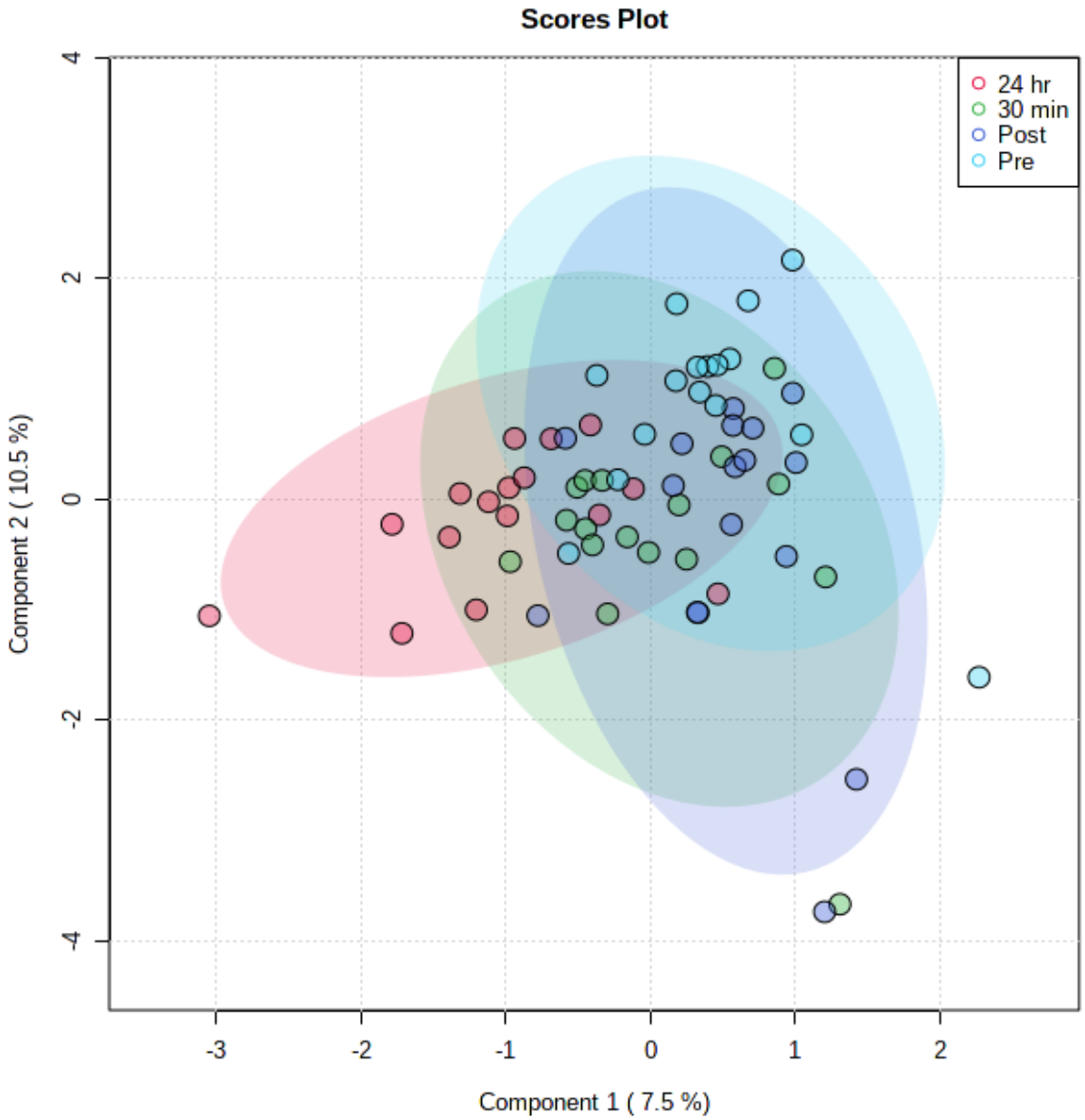
When comparing ketone salt supplementation to the placebo, leucine and valine were significantly upregulated while isoleucine decreased with supplementation. Phenylalanine which was downregulated following exercise was similar between the ketone salt supplementation and placebo. Tyrosine, a ketogenic amino acid that occurs downstream in the phenylalanine pathway had similar concentrations throughout the exercise sampling period but significantly downregulated following ketone salt supplementation. Based on the concentrations obtained for phenylalanine and tyrosine, no trend between these two metabolites can be determined following exercise or supplementation. Other metabolites that were significantly upregulated post-exercise included 4-aminobutyrate, betaine, erythritol, glycerol, and histidine which have varied roles in metabolism.

Multivariate Analyses

Multivariate analyses compared metabolite concentrations across sessions 2 and 3 for the four sampling times and between ketone salt supplementation versus placebo. The PCA for the four sampling times accounted for 47% the total variation explained using principal components 1-5. Of those 26% percent of the variation occurred in components 1 and 2 as shown in the score plot (Figure 5). The metabolite concentrations did not clearly discriminate pre-exercise, post-exercise, 30 min post exercise, and at 24 h post exercise. However, a slight trend in downregulation was noted at 24 h post exercise for the metabolites. For PLS-DA and OPLS-DA supervised models, score plots for the two models showed greater visual separation with PLS-DA and OPLS-DA but total variation for principal components 1 and 2 were low at 18% and 19%, respectively. Further, the Q^2 coefficient was low at 0.01 and permutation $p = 0.038$ for PLS-DA and for OPLS-DA the Q^2 value was -0.168 and permutation $p = 0.251$ (Figure A7 and Figure A8). The Q^2 coefficient did not support cross-validation of the two models even

Figure 5

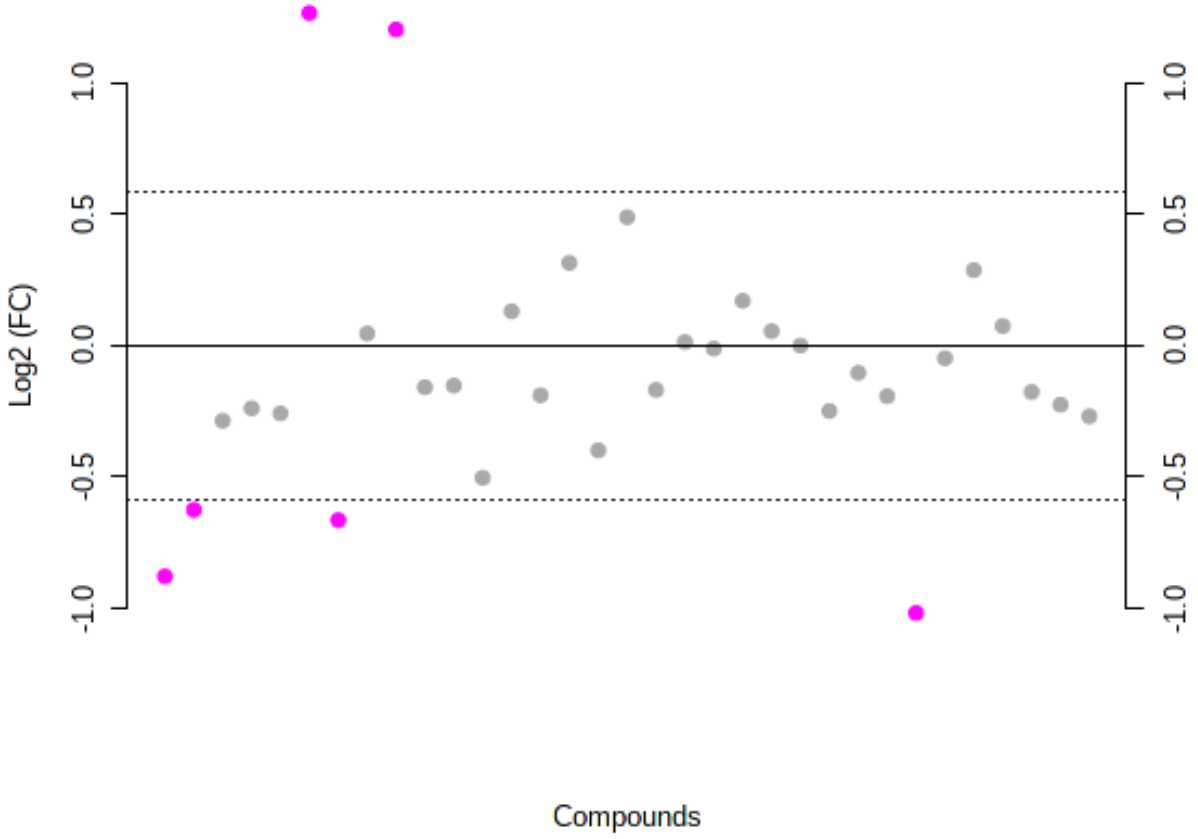
PCA Score Plot (Ketone Salt Sampling Times)



Note: Score plot of PCA model for principal components 1 and 2 explaining variance between three sampling periods pre-exercise, post-exercise, 30 min post exercise and 24 h post exercise.

Figure 6

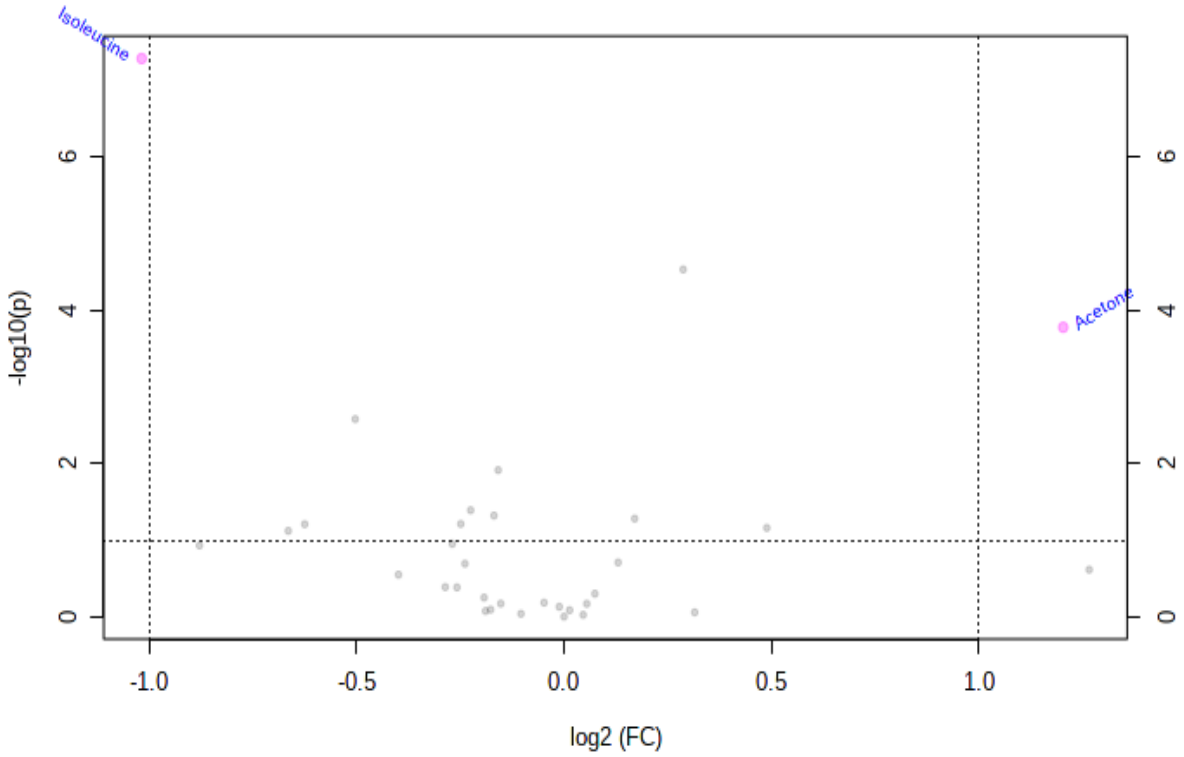
Fold-change (Ketone Salt Versus Placebo)



Note: Fold-change analysis used the threshold level of 1.5. The red circles are from six metabolites represent features above the threshold. Note the values are on log scale, so that so that same fold-change (up/downregulated) will have the same distance to the zero baseline.

Figure 7

Volcano Plot (Ketone Salt Versus Placebo)



Note: Important features selected by volcano plot using the fold-change threshold (X-axis) 1.5 and t-tests threshold (Y-axis) $p \leq 0.05$ for ketone salt versus placebo mean metabolite concentration values. The red circles represent the metabolites isoleucine and acetone above the threshold indicating significance metabolite changes.

though permutations were significant for PLS-DA. VIP values of individual metabolite were not provided since none of the models were significant. Univariate analysis was conducted comparing metabolite concentrations following ketone salt supplement versus placebo. To evaluate individual metabolite concentration variations during the study, a FC analysis with a

threshold of 1.5 for ketone salt versus placebo/variable (each metabolite), showed upregulation of the two ketone bodies β-hydroxybutyrate and acetone (Figure 6).

The four metabolites that downregulated were isoleucine, 2-aminobutyrate, 4-aminobutyrate, and 2-hydroxybutyrate. To verify the FC results, a volcano plot analysis was conducted for the paired variables that compared results from the t-test with $p \leq 0.05$ on y-axis with those from FC on the x-axis (Figure 7). The volcano plot further confirmed that acetone was significantly upregulated, and isoleucine downregulated following ketone salt ingestion but the other four were excluded.

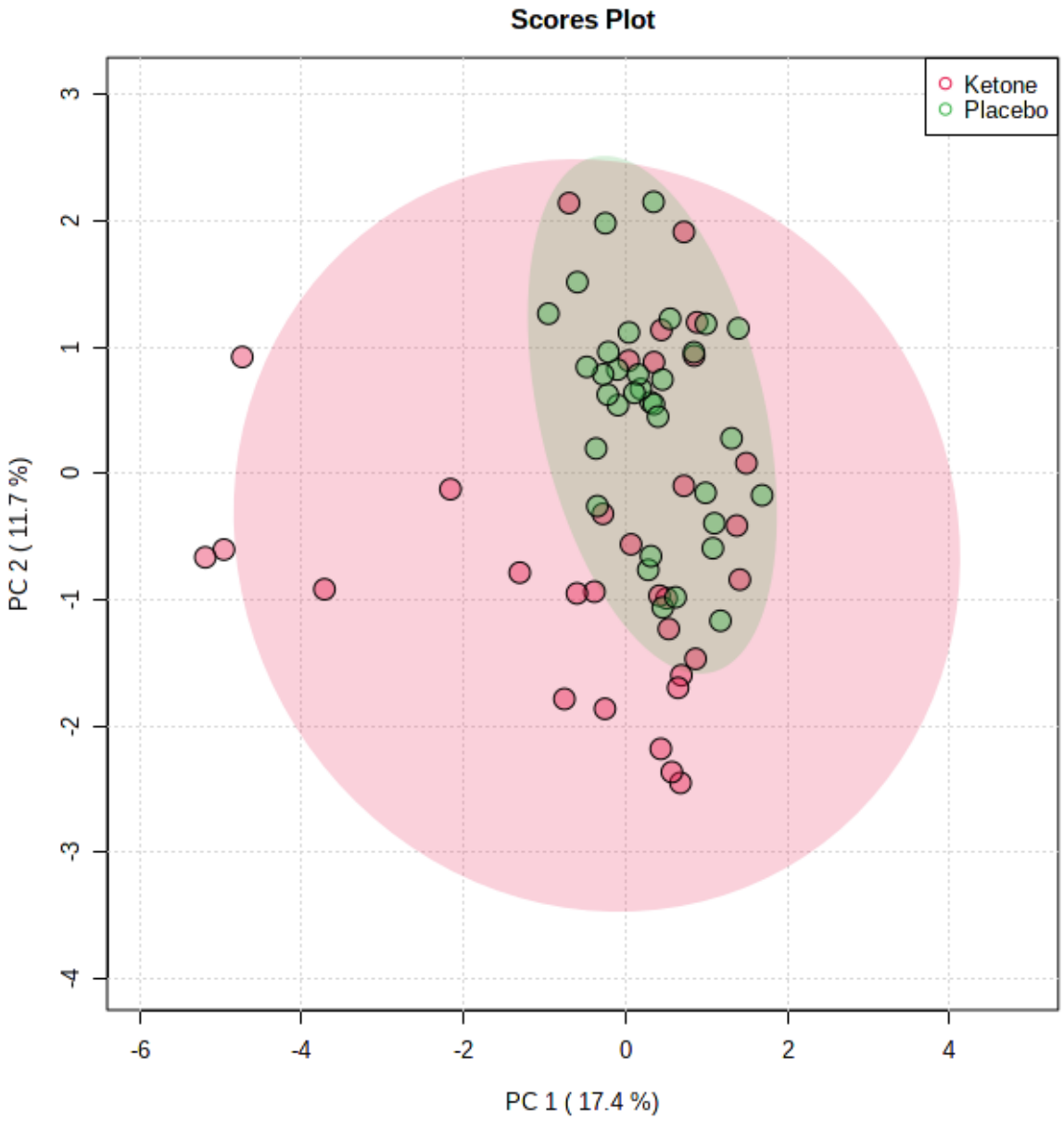
Multivariate analysis comparing ketone salt versus placebo using the PCA model determined that principal components 1 through 5 accounted for 53% of the total variation. Of those components 1 and 2 accounted for 29.1% of the total variation in the score plot (Figure 8). The score plot for ketone salt showed a slight downward trend but most metabolite concentrations strongly clustering with the placebo. The model overall did not support two distinct metabolite groupings based on mean concentrations between ketone salt and placebo.

Further visualization of the ketone salt versus placebo comparison was conducted using supervised PLS-DA and OPLS-DA models. Results from the cross-validations for PLS-DA determined that the model was not supported where Q^2 coefficient (0.225) was below the minimal acceptable range for validation even though the permutation value was $p < 0.001$ (1000) (Figure A9). When the OPLS-DA model was run the Q^2 coefficient (0.243) was also below minimal even though the permutation value $p < 0.001$ (1000) was significant (Figure A10). The Q^2 coefficient values for both models were below minimal acceptable range indicating that the metabolites were not impacted by ketone salt supplementation compared to the placebo. VIP

scores data for individual metabolites were not relevant since the Q^2 coefficient values were not significant.

Figure 8

PCA Score Plot (Ketone Salt Versus Placebo)



Note: Scores plot between the selected PC for the metabolites and concentrations comparing groups between metabolite concentrations for the ketone salt versus placebo supplementation.

Discussion

The total number of metabolites consistently identified in this study were 38 while 8 others were detected irregularly or at low concentrations with ^{1}H NMR. Exercise and ketone salt supplementation had a significant impact on 13 of the metabolites analyzed, but these results did not show direct benefits for reducing transient OS. However, indirect affects by significant metabolites are discussed below.

As previously reported transient ROS from poor nutrition and lack of exercise support chronic OS that can lead to advanced atherosclerosis, which can eventually cause sudden cardiac events during intense physical activity by FF (Huang et al., 2013). Any methods that can be recommended to combat chronic OS would be beneficial for first responder cardiovascular health. The present study showed that ketone supplementation supported significant upregulation of β-hydroxybutyrate and acetone immediately following exercise potentially supporting reductions of OS and cellular repairs of damaged tissues (Rojas-Morales et al., 2020). Ketones such as β-hydroxybutyrate were reported to lower OS, increase antioxidant activity and scavenging of free radicals in male rats (Greco et al., 2016). In that study, the ketogenic diet attenuated OS by increasing transcription of proteins forming antioxidants, NAD dehydrogenase quinone 1, and superoxide dismutase 1 and 2. During that study the ketogenic diet supported greater mitochondrial Complex I – III functionality compared to the controls resulting in greater mitochondrial oxidative phosphorylation suppressing ROS formation. In another study using male rats, antioxidant levels trended higher using a short-term ketogenic diet than standard chow food with ketone salt supplementation (Kephart et al., 2017). Furthermore, upregulation of β-hydroxybutyrate was reported to reduced OS by inhibiting H_2O_2, nitric oxide expression and histone deacetylase inhibition (Kong et al., 2017). Specifically increased ketone levels caused

upregulation of Forkhead box O3 (FOXO3) transcription factor that mediates many positive cellular functions, superoxide dismutase and catalase in rats. In the present study, univariate analysis using FC and volcano plot analyses comparing ketone salt versus placebo metabolite concentrations confirmed that β-hydroxybutyrate and acetone significantly increased following the treadmill exercise protocol and ketone supplementation. The results from the current ^{1}H NMR study suggests potential positive benefit of exercise and ketone salt ingestion for lowering transient OS following exercise.

The BCAAs stored in skeletal muscles are generally catabolized following exercise from reduced blood glucose levels for maintaining energy levels. With the need for ATP production from exercise activity, the BCAA can be converted to final products acetyl-COA and succinyl-COA for use in citric acid cycle or increased leucine for protein synthesis for muscle hypertrophy and repair following exercise (Mitch & Golberg, 1996; Holecek, 2018). In this study, the three BCAAs concentrations were lower after exercise indicating their potential oxidation for ATP or protein synthesis.

The ketone bodies acetone and β-hydroxybutyrate were greater following the ketone salt and exercise protocol. While exercise itself may have increased energy needs resulting in greater adipose catabolism for eventual conversion to acetyl-COA for use in citric acid cycle (Nie et al., 2018; Han et al., 2020). The exogenous supplementation was the most likely source of the increased acetone and β-hydroxybutyrate concentrations following exogenous supplementation

As shown in Table 3, 4-aminobutyrate significantly increased immediately after performing the exercise protocol by the FF. 4-aminobutyrate, formed from glutamate is a neurotransmitter inhibitor that supports reduced biological stress (Shelp et al., 2017). This mechanism is believed to be induced by increased respiration signal formation of succinic

semialdehyde reductase during abiotic stress activities related to OS injury associated with cardiovascular disease. This metabolite was reported to inhibit H_2O_2 induced stress indirectly as a signaling mechanism associated with cytokinin (NF-κB) inhibition (Jin et al., 2013; Di Lorenzo et al., 2016; Zhu et al., 2018). In the current study, the immediate significant upregulation by 4-aminobutyrate may be a response by the body to alleviate transient OS but further research must be conducted to support this hypothesis.

Very high levels of BCAAs have been reported to facilitate increased inflammation leading to atherosclerosis and OS causing mitochondrial (Zhenyukh et al., 2017) and endothelial dysfunction (Zhenyukh et al., 2018). However, contrary to these studies, normal blood plasma levels of BCAAs are found to reduce OS by signaling induction of mitochondrial biogenesis providing beneficial effects on protein synthesis and insulin resistance (Fukushima et al., 2007). In the FF study, levels of isoleucine and valine were downregulated immediately following exercise with both trending back to pre-exercise levels within 24 h post exercise. The reduction in these two BCAAs following exercise may contribute indirectly to reduced OS while leucine supports signaling for muscular hypertrophy through protein synthesis. Leucine was significantly upregulated post-exercise and decreased at 30 min and 24 h post exercise to pre-exercise levels in the current study. Transient differences were also shown for ketone salt supplementation versus placebo indicating that leucine was significantly greater with supplementation. Lactate which is known to increase transient OS following exercise was reported to initiate signaling downstream for antioxidant formation offsetting the transient OS effects following exercise (Tauggenberger et al., 2019). Based on previous studies, the transient increases in leucine may have contributed to some level of transient OS suppression.

Histidine was significantly downregulated 30 min post-exercise and recovered to pre-exercise levels at 24 h post-exercise. Histidine can be converted to glutamate leading to formation of glutathione, which can act as a metal ion chelator, and a scavenger of ROS and NOS (Holecek, 2020). Betaine, a trimethyl derivative of glycine was significantly upregulated post-exercise and 30 min post exercise but returned to pre-exercise levels at 24 h post exercise. Betaine is a beneficial chemical that can reduce lipid accumulation and indirectly suppress OS that supports necessary transient inflammation and apoptosis (Deminice et al., 2015). This metabolite was reported to increase levels of nonenzymatic antioxidants, S-adenosylmethionine (SAM) and methionine (Zhang et al., 2016). However, histidine and betaine concentrations were similar to the placebo following ketone salt supplementation.

Glucose levels were similar pre- and post-exercise from the blood plasma samples and supplementation indicating homeostasis. Whereas a transient significant increase in glycerol during the current study could reflect lipolysis of triglycerides from adipose tissues releasing glycerol. The significant upregulation of glycerol occurred post-exercise and 30 min later but returned to normal within 24 h post exercise. With glucose levels maintained throughout the study, energy production may have supported a transient increase in lipolysis during exercise utilizing free fatty acids through β-oxidation forming acetyl-CoA. The acetyl-CoA was transported into the citric acid cycle and then to electron transport chain for ATP production. However, there were no changes in carnitine levels that would have suggested increased need as a carrier for transport of free fatty acids into the mitochondria prior to β-oxidation. Adipose lipolysis was possible due to transient energy needs during exercise activity by the FF but could not be confirmed in the current study.

Conclusion

In conclusion, ketone salt supplementation and exercise supported transient upregulation of important metabolites in the FF such as β-hydroxybutyrate, 4-aminobutyrate, and from three BCAAs for reducing OS and improved cardiovascular health. These specific metabolites identified from the ^{1}H NMR analyses may provide a holistic defense mechanism for reducing transient OS suggesting the need for further study of these metabolites following physiological stress. Furthermore, it is believed that results from ^{1}H NMR may suggest metabolic cardiovascular benefits to FF using ketone salt supplement and exercise relate to transient OS.

CHAPTER VI

OVERALL CONCLUSIONS

Research to improve cardiovascular health of FF was previously conducted in three separate studies to determine if nutritional practices, exercise, and supplementation protocols would reduce transient OS. In those studies, blood plasma targeted antioxidants or OS associated metabolites were used to evaluate the different treatments in the three investigations. Subsamples of the blood plasma for all three studies, stored over long periods of up to 2 years at - 80°C, were analyzed using ^{1}H NMR to further identify untargeted metabolites that support metabolic changes associated with transient OS and cardiovascular health.

Results from three FF studies consistently identified 34 to 40 metabolites that were limited to the plasma samples. While no endogenous antioxidants associated with OS were identified in the current investigation several ^{1}H NMR detected metabolites have been reported to indirectly reduce OS levels, especially the ketone body β-hydroxybutyrate, BCAAs isoleucine, leucine and valine which are important for improved cellular functions and cellular health (Figure B1). The significant upregulation of lactate following exercise is another example of a metabolite supporting molecular downstream translational signaling for antioxidant formation.

The three blood plasma studies determined that lactate, β-hydroxybutyrate, isoleucine, leucine, and valine levels were significantly impacted following combinations of exercise protocols, a ketone salt supplementation and CRD. Endogenously formed lactate was reported to be responsible for 5-20% of all ATP synthesis, while ketones, glucose and acetate, and pyruvate

plus BCAAs contributed 5-15%, 20-30%, and less than 5%, respectively (Gibbs et al., 2018). These metabolites become increasingly more important when glucose levels are low from reduced carbohydrate diets, as shown in many other ketogenic studies. All five metabolites can be catabolized forming acetyl-COA.

In two of the current ^{1}H NMR studies, lactate levels were significant greater immediately after physical activity that supported some level of anerobic metabolism. Lactate serves as an indirect biomarker of anaerobic metabolism following physical activity or resistance exercise associated with muscle fatigue (Ishii and Nishida, 2013). Anerobic metabolism at the pyruvate level initiates lactate formation downstream as oxygen and glucose supplies become limited, suppressing acetyl-COA formation. The FF study using oral ketone salt supplementation, followed by intense acute exercise protocols (± heat) was the principal factors responsible for increased transient lactate levels, whereas the 28-day-CRD study using FF did not include an exercise protocol just prior to the blood sampling sessions and resulted in similar concentrations of lactate.

The significant variations in concentrations of three BCAAs were partially due to the ingestion of β-hydroxybutyrate supplement in the ketone salt study and endogenous formation of β-hydroxybutyrate from 28-day-CRD. Fluctuations in isoleucine, leucine, and valine may be attributed to energy needs met by endogenously maintaining glucose homeostasis in the blood while supporting metabolic processes for reducing oxidative stress. The exogenous and endogenous sources of energy synthesis during the 28-day-CRD included protein turnover from increased uptake in protein rich foods during the study and from skeletal muscle catabolism (Brosnan & Brosnan, 2006). Protein turnover is believed to be ~ 18 kJ (4.3 kcal)/kg of body weight or 20% of basal metabolic rate (Institute of Medicine, 1999). However, muscle is not

considered glucogenic and only when other sources of energy are being depleted is protein degradation necessary for glucose homeostasis to maintain muscle energy and brain functions. The increase concentrations of isoleucine, leucine and valine occurred following protein degradation from muscular tissues were transported from blood into cells for conversion into acetyl-COA and oxidized through the citric acid cycle supporting ATP synthesis. The endogenous sources of protein from skeletal muscle are processed through the ubiquitin-proteosome proteolytic pathway (Mitch & Goldberg, 1996; Tiao et al., 1997), forming acetyl-COA. The three BCAAs provide different metabolic functions as leucine is ketogenic for ketone body formation, valine is glucogenic for energy production and isoleucine is utilized in both pathways (Brosnan & Brosnan, 2006).

The ketone bodies, acetone and β-hydroxybutyrate are formed exogenously from food and endogenously from adipose tissue triglycerides. The triglycerides are degraded to glycerol and free fatty acids to meet energy demands, especially during periods of reduced sugar and carbohydrate intake (Han et al., 2020). In the 28-day-CRD and ketone salt supplementation studies the concentrations of β-hydroxybutyrate and acetone were significantly greater but similar to those in the curcumin study. To form ketone bodies the adipose tissues triglycerides are degraded to free fatty acids and glycerol. The free fatty acids are transported into liver where they form two acetyl-COA molecules or acetoacetate and other ketone bodies downstream, depending upon metabolic needs. Acetoacetate forms acetone downstream when in excess and then is removed from the respiratory airways as it is oxidized to CO_2 and by urine with these two accounting for 2 to 30% of acetoacetate removal (Reichard et al., 1979). Acetoacetate and β-hydroxybutyrate can be transported by plasma into the cells to maintain energy homeostasis

(Newman & Verdin, 2017). Within the cells, β-hydroxybutyrate can be converted from acetoacetate back to acetyl-COA for ATP production.

Overall, these current data suggest that the nutritional and exercise protocols may be beneficial for improving FF cardiovascular health but leave many unanswered questions. Further research is recommended, especially evaluating ketogenic supplementation or dietary approaches for enhancing increased ketone body formation through CRD, or other fasting measures coupled with acute or chronic exercise supporting lactate upregulation (Figure B1). However, to justify using a NMR platform in OS research, future studies should include temporal analyses beyond stored plasma samples to include whole blood for detection of antioxidants and lipids identifying a broader range of untargeted metabolites that are important biomarkers. Furthermore, multivariate analyses with larger blood sample sizes would provide better separation of treatment effects using metabolite concentration variations enabling individual metabolites to be discussed using VIP results obtained during PLS-DA and OPLS-DA model analyses. This comprehensive approach for metabolite detection and analyses would increase our understanding for using specific supplementations and dietary interventions with exercise for mitigating first responder OS-related activities leading to chronic inflammation causing metabolic disease.

Limitations

The primary limitation in the overall study was the inability to determine important metabolites and concentrations that are only accurately detected within the first 24 hours of blood collection using ^{1}H NMR analyses Some metabolites will bind to proteins in blood plasma within several hours after sampling or catabolism occurs. The stored blood plasma from the three studies limited the total number of metabolites that could be detected due to their age. A

previous study suggested that concentrations of amino acids in stored samples may be impacted over time. Glutathione, the most abundant and critical antioxidant in the body, may not be detectable using NMR after first few hours from sampling. This is also true for other important biomarkers such as ascorbate that are critical antioxidants to reducing hydroxy radicals. Research on blood storage is somewhat contradictory regarding the impacts of storage timing but generally first 24 h following sampling is when the most rapid metabolite concentration changes occur, as is the case with glucose and lactate. In general, mean glucose levels were lower than normal throughout the three studies while lactate was similar or slighter higher than expected in blood plasma. However, research on storage limitations for the majority of the other metabolite concentration variations over time is limited.

Secondly, more recent studies suggest that whole blood provides greater detection potential for quantifying great numbers of metabolites with more accurate concentration determination than do serum and plasma. With whole blood additional metabolites such as the major antioxidants could have been compared to the treatments for the three FF studies using ^{1}H NMR. In addition, other metabolites present in whole blood such as NAD+, NADH, NADP+ and NADPH coenzymes and their ratios are supportive biomarkers that indicate mitochondrial and cellular dysfunction or health as they are important in signaling in metabolic reactions and in energy production, all impacting OS. For untargeted metabolomic research related to human cardiovascular health, blood samples should include whole, plasma, and serum extraction protocols for assessing OS-related metabolites, lipolysis or lipogenesis using ^{1}H NMR.

Future Research

To further determine usefulness of ^{1}H NMR for OS and cardiovascular health studies, new investigations evaluating whole blood, serum, and blood plasma immediately after sampling versus storage intervals should be conducted. Based on recent literature, whole blood will provide greater information and data collection should include bracketing of storage timing to determine accuracy or limitations of evaluating blood during short and long-term storage samples. Secondly, studies should be developed to determine if ^{1}H NMR data can stand alone or whether those results must be coupled with traditional bench top data collection that evaluates targeted metabolites as in the three previous studies (McAllister et al., 2018, 2019; Waldman et al., 2020). This dual approach could be used to better define OS stress and cardiovascular benefits from different physical stressor protocols, dietary challenges and/or supplementations. Even though multivariate analyses are the most used methods for metabolomic studies to date, the analyses from present studies were of limited value for determining OS benefits based on concentration data, whereas multivariate analyses have the strongest application for variation in total numbers of metabolites using the models PCA, PLS-DA and OPLS-DA. Further research using metabolite concentration data with these models should be done but with much larger sample sizes.

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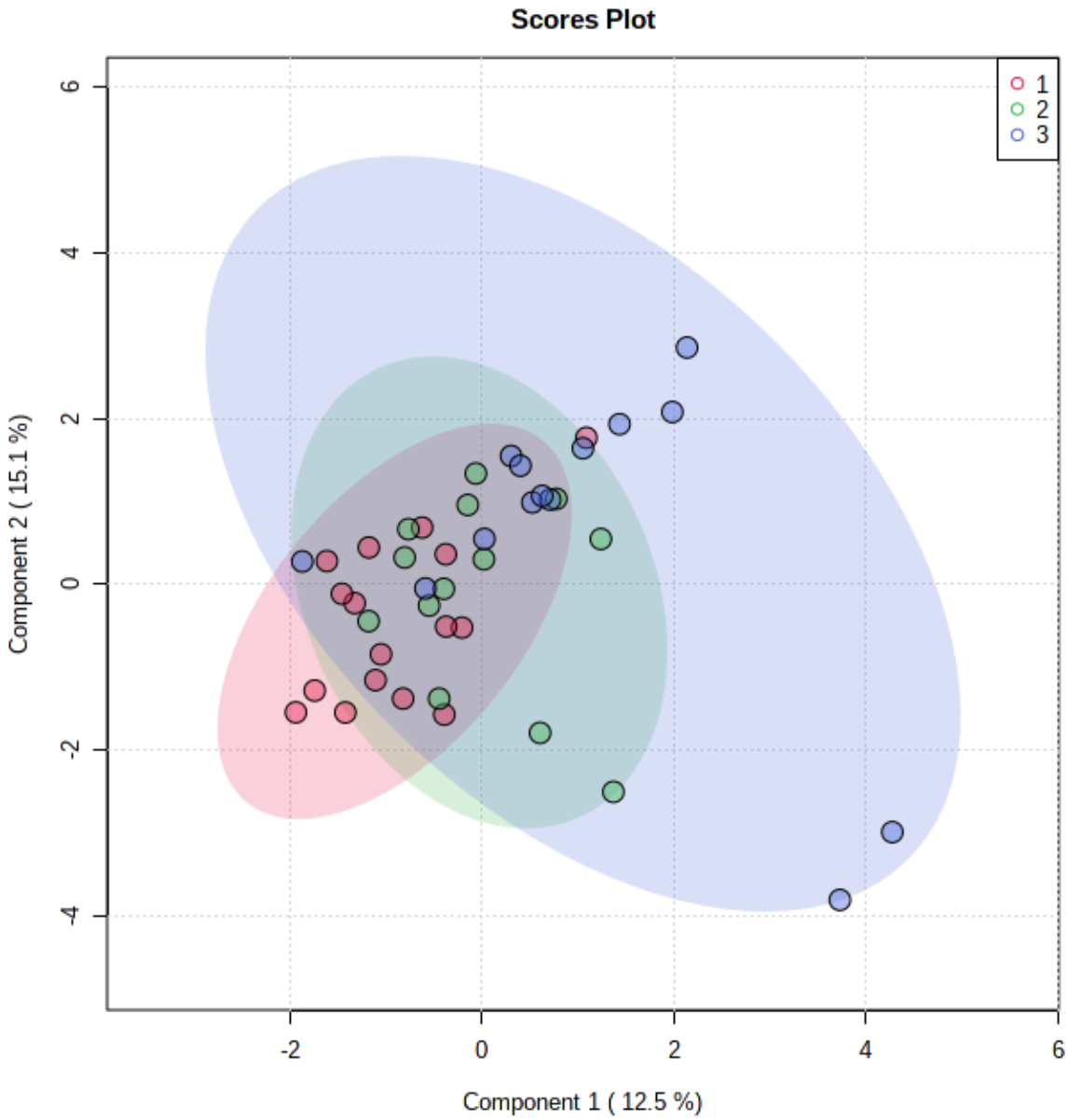
APPENDIX A

MULTIVARIATE ANALYSIS

Figure A1

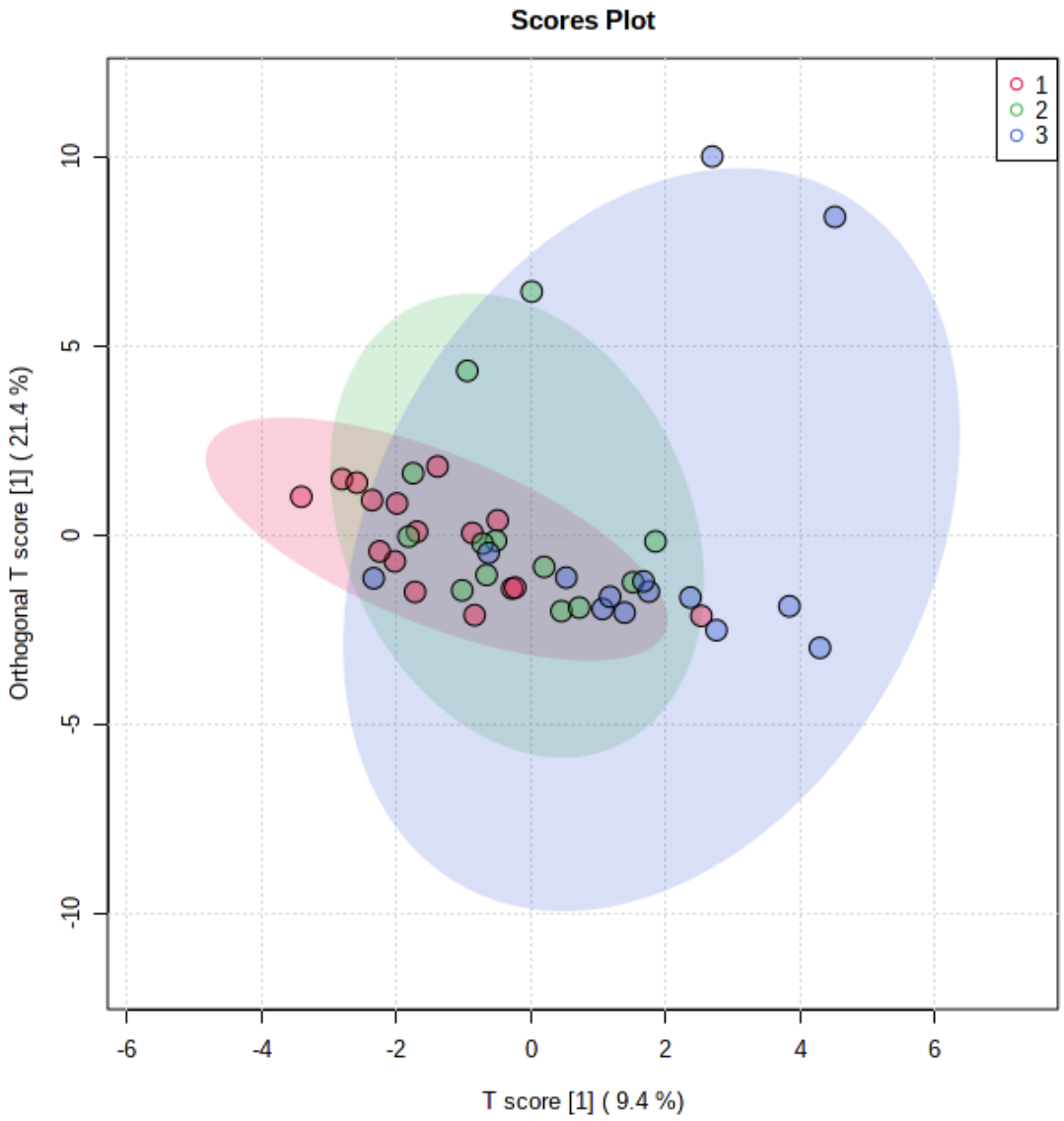
PLS-DA Score Plot (CRD)



Note: Metabolite concentration distributions for the three sessions (trials 1, 4 and 7) of firefighter's blood plasma with % variance in brackets. Session 1= familiarization, 2= baseline, and 3= post-28-day carbohydrate restricted diet.

Figure A2

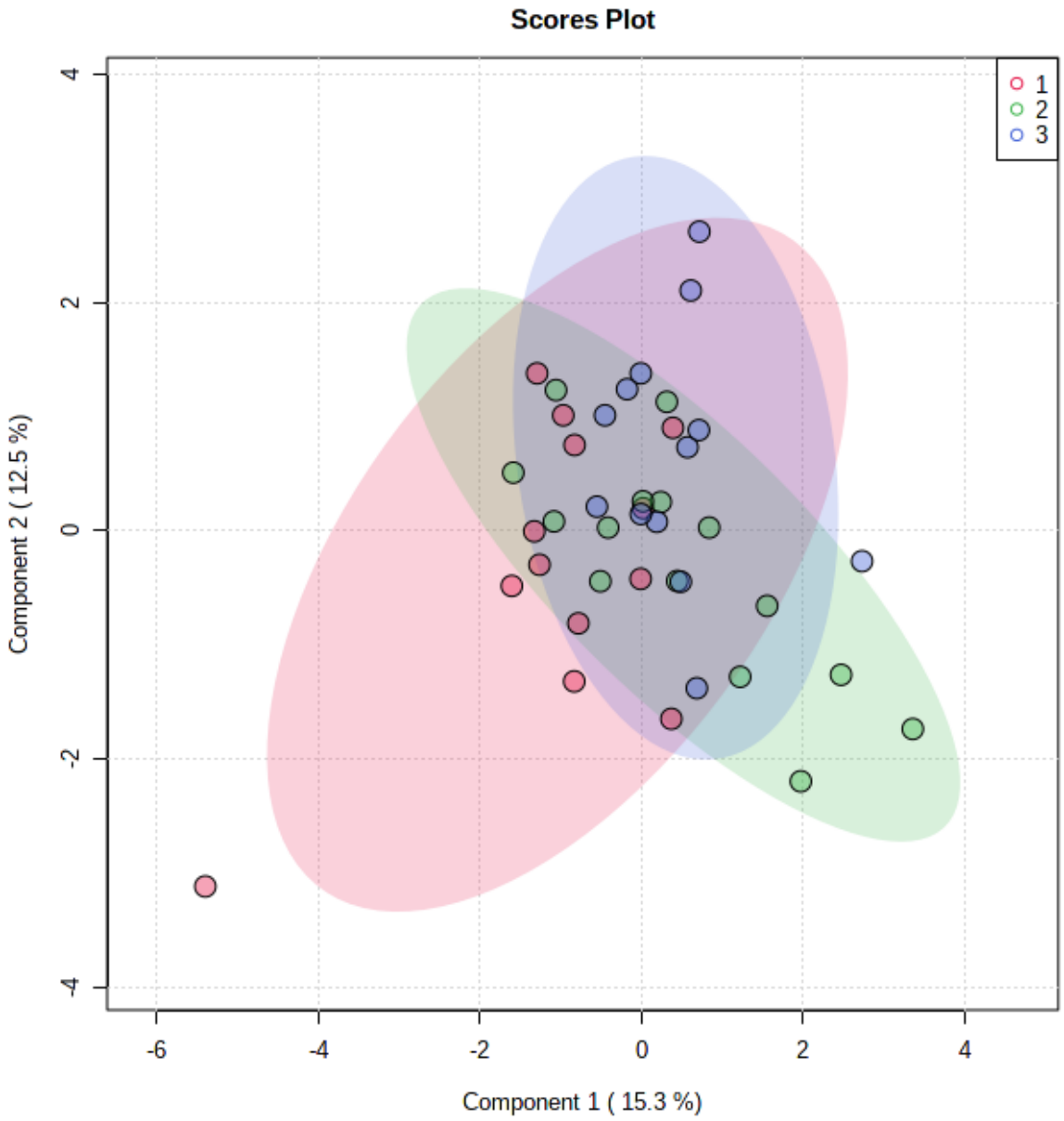
OPLS-DA Score Plot (CRD)



Note: Metabolite concentration distributions for the three sessions (trials 1, 4 and 7) of firefighter's blood plasma with % variance in brackets. Session 1= familiarization, 2= baseline, and 3= post-28-day carbohydrate restricted diet.

Figure A3

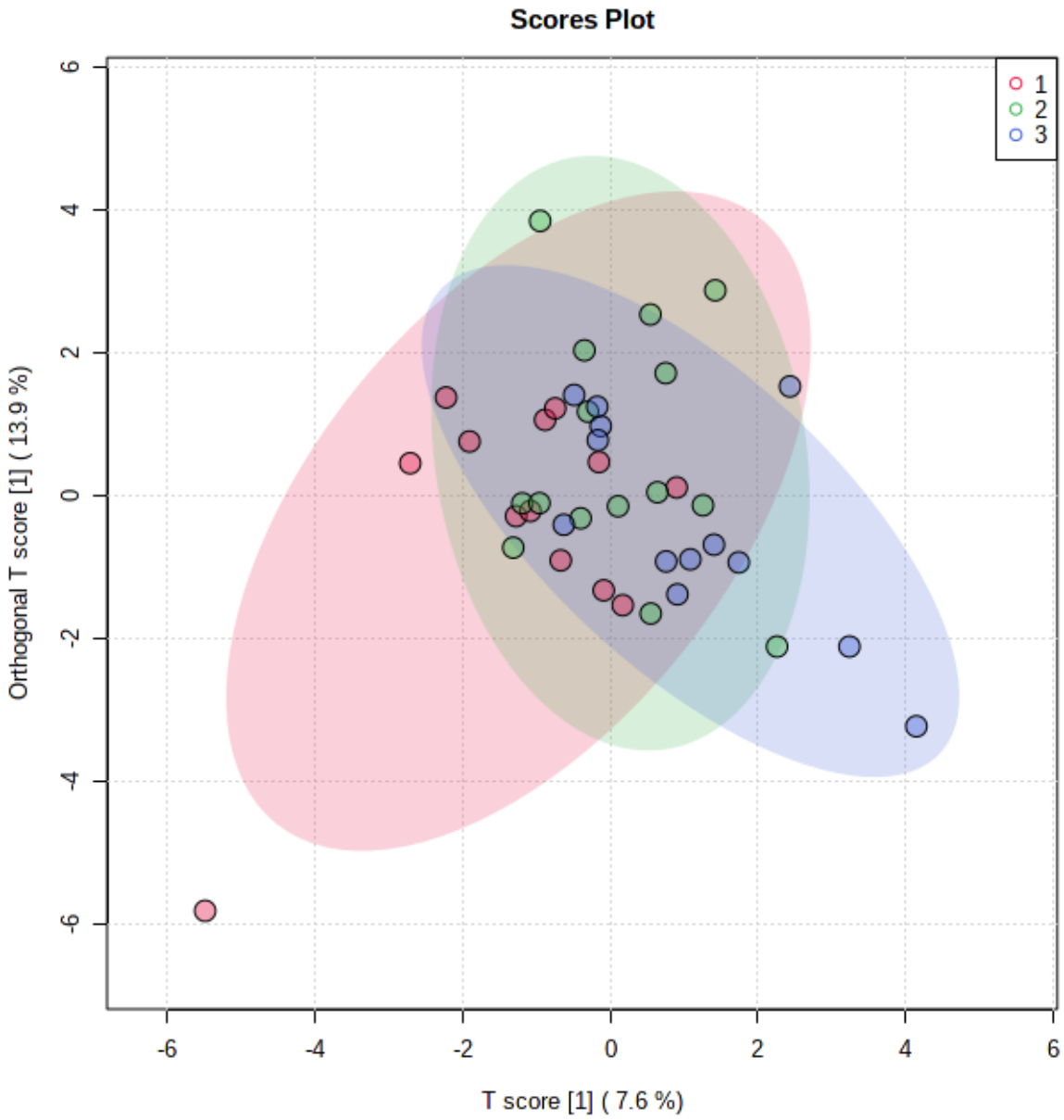
PLS-DA Score Plot (Curcumin Sampling Times)



Note: Score plot of PLS-DA model for principal components 1 and 2 explaining variance between three sampling periods pre-heat exercise (1), post-exercise (2) and 30 min after exercise (3).

Figure A4

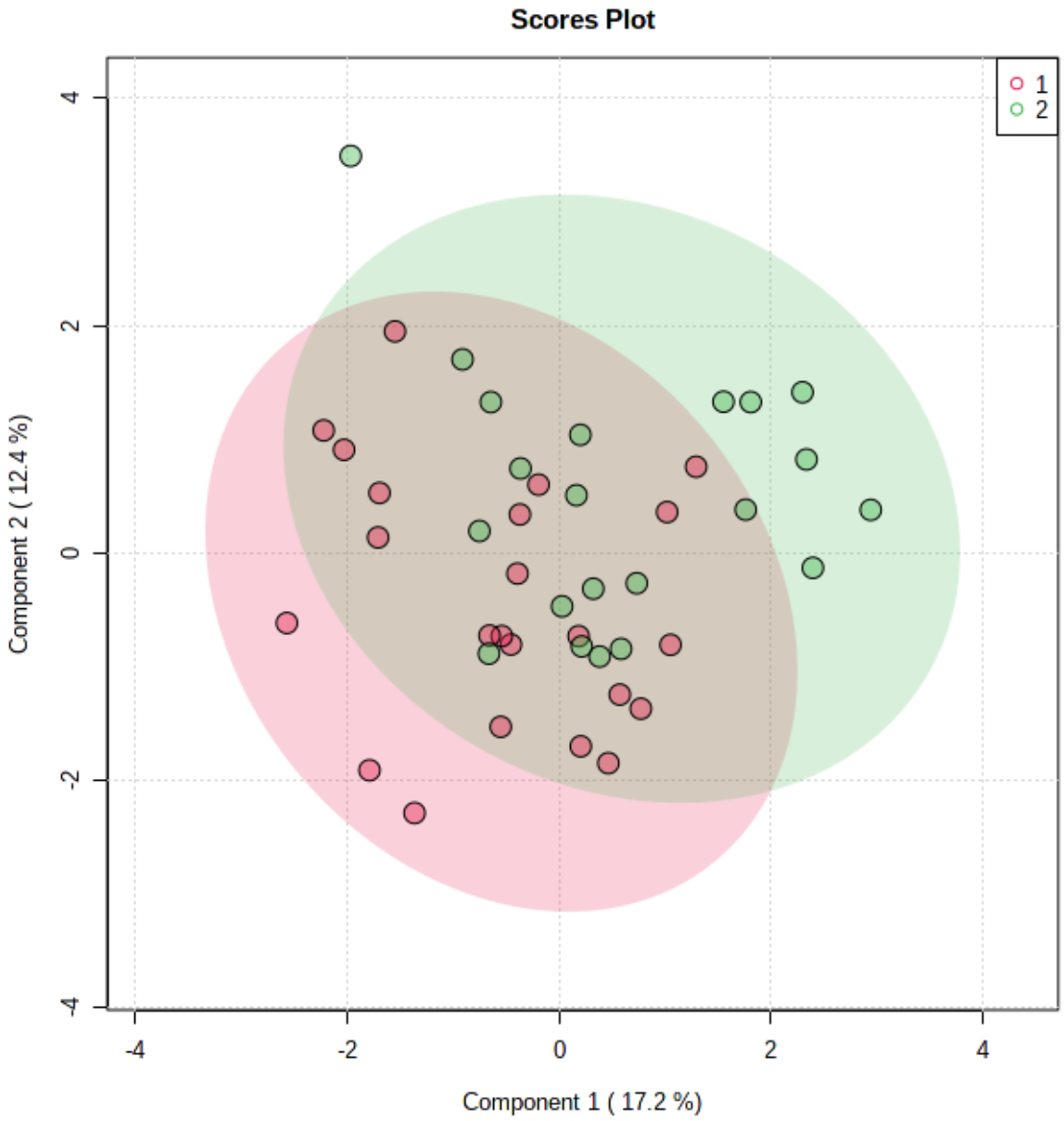
OPLS-DA Score Plot (Curcumin Sampling Times)



Note: Score plot of OPLS-DA model for principal components 1 and 2 explaining variance between three sampling periods pre-heat exercise (1), post-exercise (2) and 30 min after exercise (3).

Figure A5

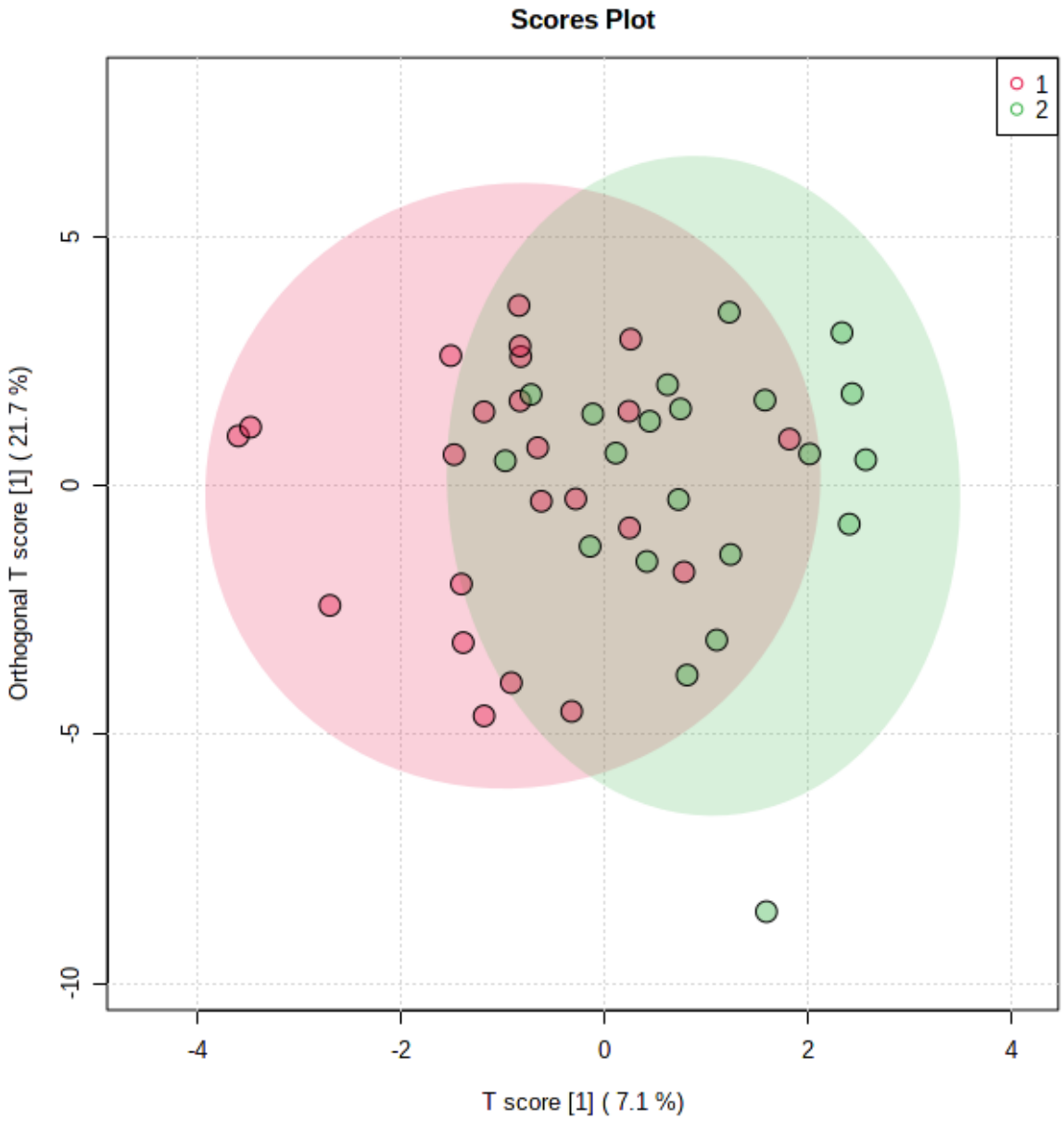
PLS-DA Score Plot (Curcumin Versus Placebo)



Note: Score plot of PLS-DA model for principal components 1 and 2 explaining variance between metabolite concentrations between (1) curcumin and (2) placebo groups.

Figure A6

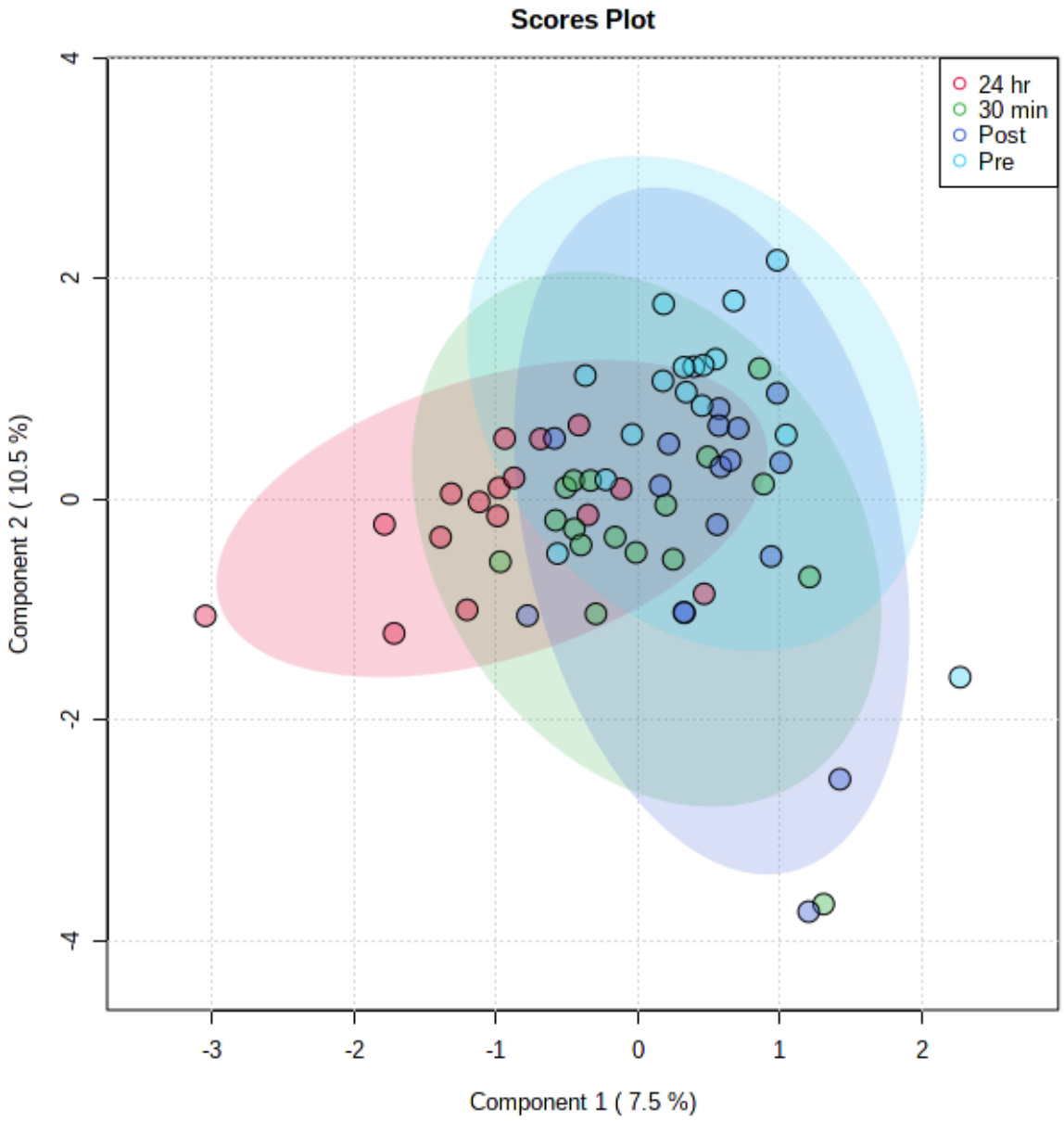
OPLS-DA Score Plot (Curcumin Versus Placebo)



Note: Score plot of OPLS-DA model for principal components 1 and 2 explaining variance between metabolite concentrations between (1) curcumin and (2) placebo groups.

Figure A7

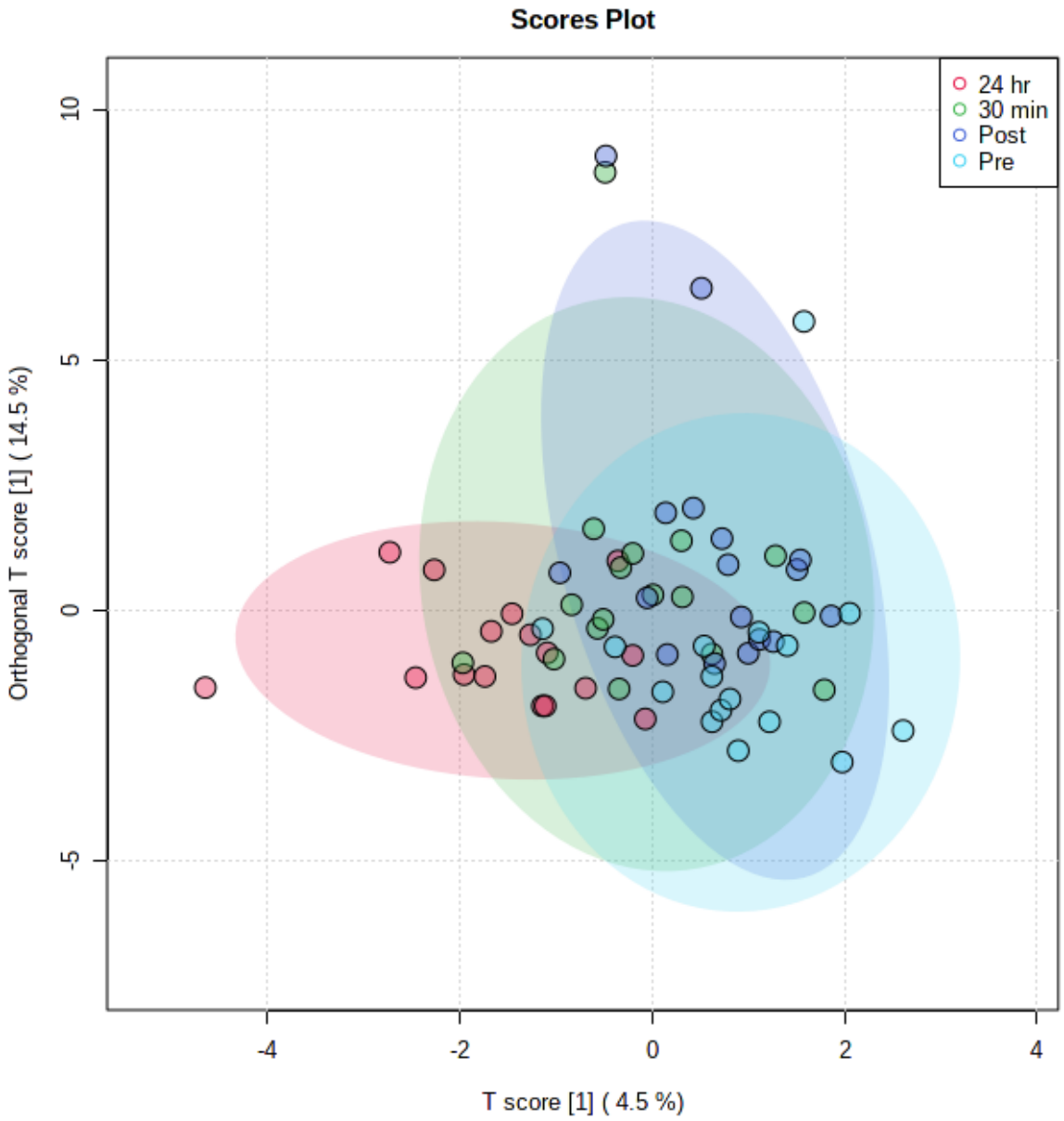
PLS-DA Score Pplot (Ketone Salt Sampling Times)



Note: Score plot of PLS-DA model for principal components 1 and 2 explaining variance between three sampling periods pre-exercise, post-exercise, 30 min and 24 h after exercise.

Figure A8

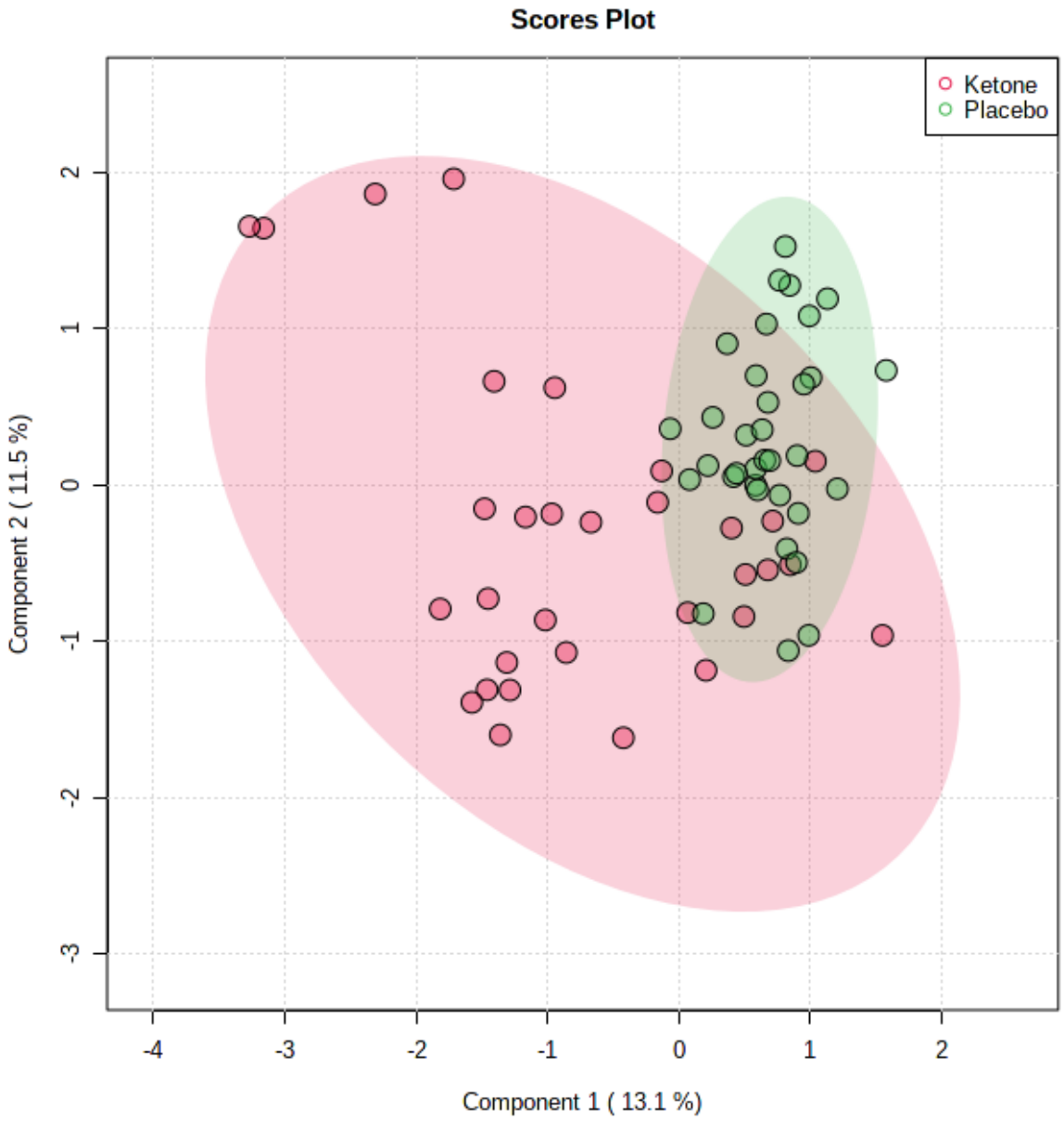
OPLS-DA Score Plot (Ketone Salt Sampling Times)



Note: Score plot of OPLS-DA model for principal components 1 and 2 explaining variance between three sampling periods pre-exercise, post-exercise, 30 min and 24 h after exercise.

Figure A9

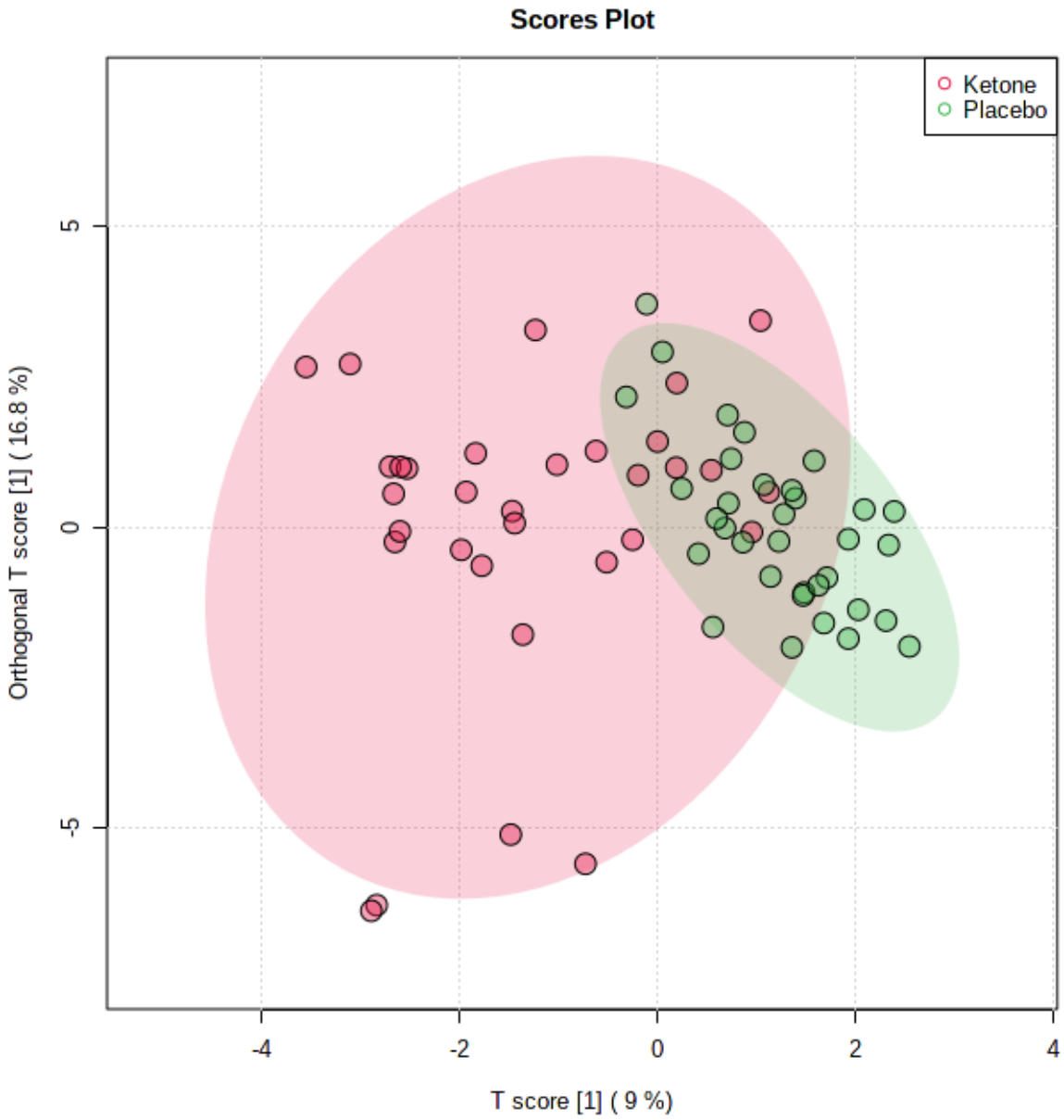
PLS-DA Score Plot (Ketone Salt Versus Placebo)



Note: Scores plot between the selected PC for the metabolites and concentrations comparing groups between metabolite concentrations for the ketone salt versus placebo supplementation.

Figure A10

OPLS-DA Score Plot (Ketone Salt Versus Placebo)



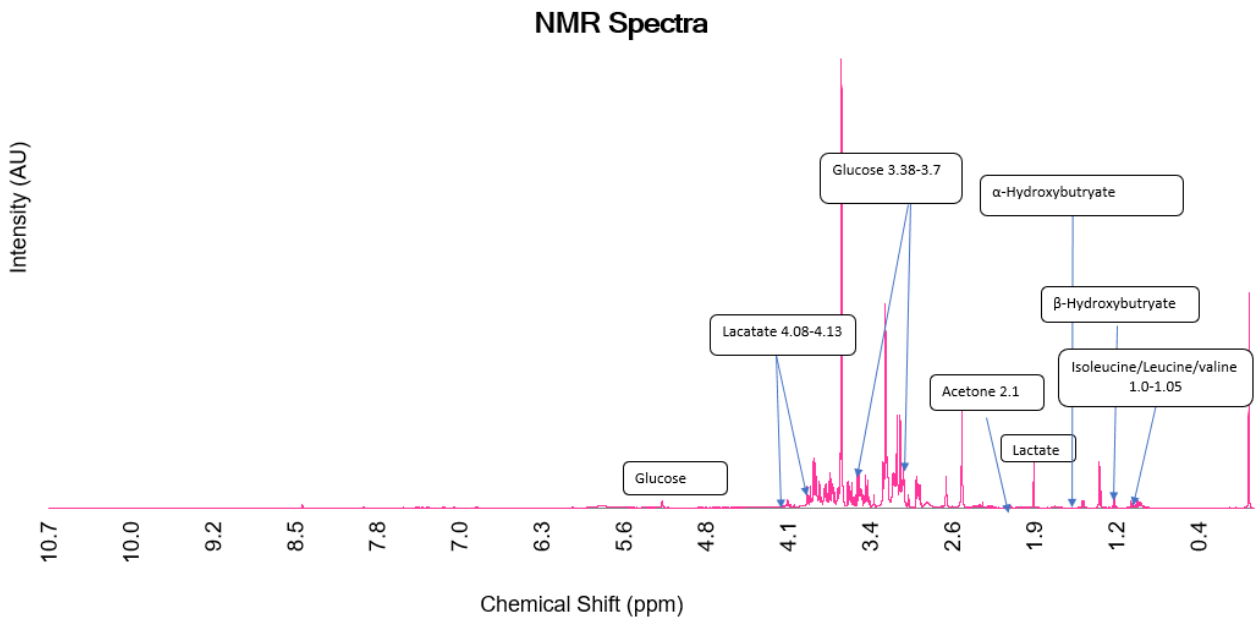
Note: Scores plot between the selected PC for the metabolites and concentrations comparing groups between metabolite concentrations for the ketone salt versus placebo supplementation.

APPENDIX B

REPRESENTATIVE ^{1}H NMR SPECTRA

Figure B1

Spectra of ^{1}H NMR Firefighter Blood Plasma Metabolites



APPENDIX C

CONCENTRATIONS OF METABOLITES FOR THREE STUDIES

Table C1

List of Metabolite Concentrations From 28-day Carbohydrate Restricted Diet Study

Firefighter Participants	Metabolite Concentrations in mM Metabolites[a] #-Session 1 Familiarization (Trial 1)												
	1	2	3	4	5	6	7	8	9	10	11	12	13
2	0.0098	0.0512	0.0331	0.0445	0.0073	0.3802	0.0976	0.1347		0.0227	0.1115	0.0145	2.0246
3	0.0995	0.1013	0.0462	0.0293	0.0093	0.3871	0.1737	0.0705	0.0553	0.0295	0.0803	0.0112	3.9349
6	0.0161	0.0871	0.0471	0.0344	0.0089	0.504	0.0705	0.0994	0.0523	0.0292	0.1040	0.0183	4.6916
7	0.0132	0.0453		0.0212	0.0089	0.5196	0.0821	0.1818	0.0781	0.0166	0.1126	0.0144	1.9964
8		0.0628	0.0395	0.023	0.0071	0.504	0.0595	0.1792		0.0085	0.1146	0.0201	1.9073
9	0.0117	0.0779	0.1099	0.0169	0.046	0.343	0.0699	0.2212	0.1136	0.0174	0.1034	0.0151	1.0284
11	0.0172		0.0085	0.0274	0.0091	0.4588	0.0879	0.0853	0.0401	0.017	0.1074	0.0146	4.4653

Table C1 (continued)

12	0.0170	0.0484	0.0610	0.0545	0.0113	0.377	0.0675	0.1926	0.1200	0.0205	0.1028	0.0170	1.9514
13	0.0120	0.143	0.0343	0.0213	0.0082	0.4692	0.0845	0.185	0.1206	0.0201	0.0991		1.9306
14		0.1033	0.0652	0.0666	0.0149	0.302	0.0737	0.1899	0.1142	0.019	0.1079	0.0136	1.7941
15	0.0155	0.0426	0.0196	0.0256	0.0074	0.309	0.0673	0.1565	0.0982	0.0217	0.1101	0.0192	1.5767
17		0.1612	0.0286	0.0252	0.0078	0.4344	0.0325	0.1371	0.0403	0.0097	0.1207	0.0145	4.3051
18	0.0134	0.0464	0.0298	0.0358	0.0152	0.4394	0.0871	0.1988	0.0797	0.0187	0.105	0.0214	2.0760
Metabolites #-Session 2 Baseline (Trial 4)													
2	0.0105	0.0797	0.0311	0.0354	0.0142	0.4758	0.0828	0.0841	0.0392	0.0208	0.1241	0.0167	4.9975
3	0.0202	0.0645	0.0231	0.0492	0.0546	0.5836	0.1316	0.1241	0.0594	0.02	0.19	0.0344	6.5892
6	0.0116	0.0792	0.0211	0.0235	0.0085	0.4467	0.0475	0.0683	0.0366	0.0179	0.0868		4.0349
7	0.0116		0.0373	0.0120	0.0046	0.3394	0.0923	0.0570	0.0442	0.0787	0.0102	0.0184	2.0142
8	0.0121	0.0404	0.0369	0.0316	0.0188	0.3816	0.0736	0.1442	0.0788	0.0125	0.1105		1.4349
9		0.0332	0.0278	0.0254		0.3281		0.1258	0.0483	0.2059		0.2095	1.9944
11	0.0111	0.0260	0.0302	0.0309	0.0113	0.2834	0.0765	0.1127		0.0237	0.0913	0.0142	1.6104

Table C1 (continued)

12	0.0181	0.0644	.	0.0365	0.0226	0.251	0.0555	0.0764	0.0267	0.0175	0.1039	0.0146	3.395
13	0.0173	0.0581	0.0215	0.031	0.0074	0.3218	0.0792	0.0664	0.0655	0.0112	0.0765		3.3052
14	0.0182	0.0711	0.0119	0.0674	0.0202	0.2417	0.0501	0.153	0.0905	0.0044	0.1005	0.0197	1.4881
15	0.0108	0.057		0.0344	0.0127	0.3042	0.064	0.0736	0.0347	0.0153	0.091	0.0184	3.6998
17	0.0122		0.0154	0.0244	0.0065	0.2765	0.0602	0.1495	0.0695	0.0037	0.0784	0.0148	1.135
18	0.0161	0.0628	0.0257	0.0423	0.0089	0.3513	0.0623	0.1179		0.0139	0.092		1.7031
Metabolites #-Session 3 28-Day-CHO (Trial 7)													
2	0.0157	0.047	0.0222	0.0492	0.0077	0.3508	0.0615	0.1166		0.0152	0.084	0.0174	1.7654
3	0.0158	0.1046		0.0286	0.0094	0.443	0.0691	0.1776	0.1258	0.0161	0.0849		1.8777
6	0.0685	0.1248	0.1708	0.0396	0.0419	0.3637	0.083	0.0873	0.0599	0.0376	0.1039	0.0206	4.5385
7	0.0188	0.0644	0.037	0.0314	0.0107	0.4302	0.0813	0.1569		0.014	0.1166	0.0173	2.2458
8	0.0171	0.0867	0.0833	0.0439	0.0218	0.4286	0.0735	0.2298	0.147	0.0273	0.1436	0.0218	2.3116
9	0.0772		0.0634	0.0358	0.0961	0.3024		0.1869		0.1421			1.6306
11	0.022	0.1496	0.1446	0.0391	0.0637	0.429	0.0908	0.297	0.1366	0.0219	0.151	0.0247	2.2426

Table C1 (continued)

12	0.0144	0.0699		0.0328	0.0277	0.3846	0.0829	0.2142	0.0839	0.0419	0.1091	0.0219	2.1751
13	0.0357	0.1204	0.0772	0.04	0.0336	0.4436	0.0933	0.2181	0.1336	0.0273	0.145	0.0198	2.206
14			0.0781	0.0836	0.035	0.4197	0.9243	0.123		0.0461			4.4547
15	0.0156	0.1101		0.0532	0.0322	0.3117	0.0619	0.1105	0.0537	0.0232	0.1328	0.0198	4.3573
17	0.0521	0.1987	0.2771	0.0456	0.0979	0.334	0.0835	0.1018	0.0561	0.0168	0.1353	0.0174	4.5878
18		0.0861	0.0908	0.0601	0.0176	0.3515		0.2244	0.1203	0.0157	0.1507		2.4309

[a]Metabolite list includes: 1) 2-hydroxyisovalerate, 2) 2-hydroxyvalerate, 3) β-hydroxybutyrate, 4) acetate, 5) acetone, 6) alanine, 7) asparagine, 8) betaine, 9) carnitine, 10) creatine, 11) creatinine, 12) formate, and 13) glucose.

Table C2

List of Metabolite Concentrations From 28-day Carbohydrate Restricted Diet Study

Firefighter Participants	Metabolite Concentrations in mM Metabolites[a] #-Session 1 Familiarization (Trial 1)												
	14	15	16	17	18	19	20	21	22	23	24	25	26
2	0.0954	0.4867	1.4873	0.1245	0.0879	0.0881	0.9604	0.1647	0.2545	0.0617	0.0166	0.0035	0.0442
3	0.0552	0.4338	1.0905	0.0951	0.0661	0.0425	0.9902	0.1318	0.1777		0.0191	0.0078	0.0491
6	0.0453	0.6033	1.3386	0.1257	0.0978	0.0892	1.6899	0.173	0.2174	0.1188	0.024	0.0036	0.0332
7	0.0992	0.6793	1.7655	0.1263	0.1069	0.0726	0.9629	0.1599	0.0178	0.0568	0.0417	0.0037	0.0176
8	0.1320	0.5746	1.9614	0.153	0.0843	0.071	1.1872	0.1676	0.0968	0.0879	0.0181		0.0285
9	0.0967	0.641	1.7465	0.117		0.0941	0.9978	0.1615		0.1369	0.0445	0.0028	0.0346
11	0.1022	0.5737	1.3595	0.128	0.0798	0.0932	0.9525	0.164	0.2275		0.019	0.003	

Table C2 (continued)

12		0.4605	1.4354	0.129	0.0841	0.0905	1.0032	0.1437	0.1927	0.0523	0.0193	0.0031	0.0245
13	0.0776	0.5545	1.5059	0.0988	0.0983	0.0451	1.16	0.1454	0.0695	0.0539	0.0396	0.0039	0.0316
14	0.0918	0.6117	1.4012	0.117	0.061	0.0585	0.6511	0.1988	0.1474	0.0608	0.0184	0.0049	0.0359
15	0.0789	0.454	1.5318	0.1178	0.0664	0.0692	0.6379	0.1484	0.1852	0.0374	0.0189	0.0024	0.0263
17		0.5783	1.4404	0.1072	0.0866	0.0789	0.7804	0.127		0.0631		0.0033	
18	0.087	0.6668	1.8221	0.1329		0.0963	1.2262	0.1383	0.1839	0.0692	0.0234	0.0047	0.0470
Metabolites #-Session 2 Baseline (Trial 4)													
2		0.5697	0.9451	0.0907	0.0512	0.0818	1.2446	0.2183	0.0759	0.0911	0.0214	0.0041	
3	0.1695	0.791	1.7699	0.1621	0.0773	0.1240	1.6343	0.257	0.4773	0.1144	0.0255	0.0045	0.0688
6	0.0723	0.4648	1.9858	0.1511	0.0805	0.0508	1.4003	0.1644	0.0866	0.0883	0.0173	0.0053	0.0547
7	0.0308	0.2726	0.6091	0.3856	0.0544	0.1003	1.5840	0.112	0.4876	0.0933		0.0038	0.0372
8	0.0931	0.5175	1.5144	0.129	0.0816	0.0842	0.9764	0.1449	0.0732	0.1147	0.0283	0.0040	0.0466
9	0.0898		1.6783	0.2839		0.4353	0.9665	0.1544	0.0865	0.1332	0.0766		0.0677
11	0.0695	0.5737	1.2191	0.1001	0.0695	0.059	0.5657	0.0651	0.2147	0.0665	0.0316	0.0021	0.0361

Table C2 (continued)

12	0.0956	0.4748	1.3248	0.1135	0.0743	0.0834	0.8936	0.1197	0.169	0.0734	0.0166	0.0029	
13	0.0735	0.3912	1.0871	0.1011	0.0685	0.0643	0.6734	0.1009	0.1644		0.0183	0.0051	0.0635
14	0.1362	0.4637	1.9367	0.174		0.0637	0.7486	0.1105	0.125	0.048	0.0279	0.0049	0.0179
15	0.1331	0.4097	1.7101	0.127	0.0619	0.0412	1.6002	0.062	0.2042	0.0596	0.016	0.002	0.0141
17		0.3576	1.1806	0.1015	0.0701	0.0479	0.6259	0.0524	0.1095	0.063	0.0102	0.0042	
18	0.1101	0.5359	2.6774	0.2044	0.0682	0.059	1.0532	0.1532		0.0543	0.0145	0.0036	0.0357
Metabolites #-Session 3 28-Day-CHO (Trial 7)													
2	0.1723	0.4443	2.6577	0.2184	0.0667	0.095	0.9817	0.139	0.2954		0.0139	0.0032	0.0406
3	0.1154	0.5038	1.6916	0.1347		0.0525	0.9395	0.1614	0.2478	0.0453	0.0178	0.0037	0.0298
6		0.6025	2.1359	0.1871	0.0806	0.0802	1.1486	0.1268	0.284	0.0583	0.0178	0.0031	0.0214
7	0.1068	0.5883	1.8503	0.1526	0.103	0.0653	0.8633	0.1638	0.2525	0.1089	0.0504	0.0031	0.0517
8	0.0899		2.0278	0.913	0.992	0.1368		0.3192	0.2501	0.1211	0.0430	0.0040	0.0512
9	0.1358	0.6738	2.1497	0.1428		0.1209	0.9266	0.1675	0.2449	0.1932	0.0242	0.0034	0.05
11	0.1491	0.5223	1.7486	0.1562	0.0923	0.1066	0.8661	0.2074		0.1391	0.0211	0.0033	

Table C2 (continued)

12	0.1082	0.7324	1.8238	0.1808	0.0871	0.1181	0.9818	0.2316	0.2956	0.1402	0.0224	0.0044	0.0229
13	0.3749	0.7065	1.7925	0.0965	0.0923	0.1578	0.8984	0.2327		1.8567	0.0264	0.0189	
14	0.144	0.7425	1.4023	0.1362	0.0716	0.0915	0.7659	0.1968	0.2382	0.0838	0.0203	0.0064	0.0187
15	0.1813	0.5601	1.47	0.1458	0.081	0.1314	0.9573	0.2184	0.278	0.0903	0.0508	0.0041	0.0144
17	0.1132	0.6732	0.6661	0.8941		0.0918	0.9204	0.1809	0.1737	2.3384	0.0682		
18	0.0941	0.6036	2.153	0.1766	0.0806	0.0872	0.7080	0.1793	0.225	0.0967	0.0422	0.0041	0.0412

[a]Metabolite list includes: 14) glutamate, 15) glutamine, 16) glycerol 17) glycine, 18) histidine, 19) isoleucine, 20) lactate, 21) leucine, 22) lysine, 23) mannose, 24) methionine, 25) N,N-dimethylglycine, and 26) phenylalanine.

Table C3

List of Metabolite Concentrations From 28-day Carbohydrate Restricted Diet Study

Firefighter Participants	Metabolite Concentrations in mM Metabolites[a] #-Session 1 Familiarization (Trial 1)												
	27	28	29	30	31	32	33	34	35	36	37		
2	0.1290	0.0067	0.0496	4.0406	0.2544	0.0082							
3	0.1328	0.0565	0.0611	3.7491	0.2237	0.0081							
6	0.2116	0.0923	0.0546	4.1582	0.296	0.0078	0.1028	0.0833		0.2579			
7	0.2807	0.0295	0.0429	4.6874	0.1427	0.0132			0.0133	0.1802	0.1399		
8	0.1593	0.0081	0.0438	3.5237	0.1909	0.0117	0.2737						

Table C3 (continued)

9	0.1603	0.0176	0.0483	7.0128	0.1641	0.0909		0.08			
11	0.1754	0.0422	0.0764	4.8831	0.1614	0.0088		0.0779		0.7654	
12	0.1221	0.0066	0.0574	2.9859	0.155	0.0166	0.1759				
13	0.1209	0.0102	0.0586	5.4757	0.0985	0.0082	0.1295				
14	0.1027	0.0079	0.0271	5.0351	0.1864	0.0117	0.1851	0.053	0.0072		0.0604
15	0.1155	0.0423		3.2677	0.0913	0.0056				0.1512	0.0844
17	0.1642	0.0049		3.4115	0.1605	0.0119	0.1917		0.0034		
18	0.2163	0.0101	0.0231	5.4364	0.1747	0.0912	0.1118		0.0112	0.1548	0.0312
Metabolites #-Session 2 Baseline (Trial 4)											
2	0.1871	0.0841	0.0705	4.9927	0.1752	0.0966	0.1586	0.038	0.0093		
3	0.2349	0.0117	0.0803	10.3039	0.4883	0.1290					
6	0.1554	0.0723	0.0412	3.4629	0.2442	0.0077					
7	0.0887	0.0309		3.616	0.2590	0.0052	0.1319				
8	0.2386	0.023	0.0420	5.2115	0.0676	0.0769	0.1444			0.1553	

Table C3 (continued)

9	02267		0.0441	4.983	0.387	0.0389	0.0921				0.4954
11	0.1222	0.0057	0.0466	3.0989	0.1205	0.0040			0.004		0.1214
12	0.1542	0.0049	0.376	3.1508	0.0583	0.0614	0.2272	0.0319			0.0246
13	0.1294	0.007	0.0402	3.7233	0.1777		0.0113		0.0054		
14	0.1258	0.0072	0.0305	4.3741	0.1299	0.0652	0.0283				
15	0.1183	0.0068	0.0381	2.7312	0.0923	0.0087					
17	0.1524	0.0062		2.1735	0.1124	0.0514					
18	0.1738	0.0395	0.0234	4.4504	0.1541	0.0071	0.1057				
Metabolites #-Session 3 28-Day-CHO (Trial 7)											
2	0.1525	0.0586	0.0268	4.3894	0.0608	0.0116				0.0964	
3	0.1576	0.0667	0.074	5.4381	0.1741	0.0829	0.1081	0.0652	0.0121		
6	0.1255	0.0069	0.0523	5.7406	0.2689	0.0156		0.0532	0.0065		0.0282
7	0.1767	0.0211	0.0515	5.6945	0.1556	0.0114	0.081	0.0788	0.0078		
8		0.1062		23.7431	0.3271						0.1356

Table C3 (continued)

9	0.2216	0.0078	0.0648	10.8604	0.3282	0.0924	0.233	0.1532			
11	0.1844	0.0073	0.032	5.8755	0.23	0.0048	0.073	0.0562	0.0094		
12	0.1557	0.0056	0.067	7.9627	0.4055	0.0213	0.3494	0.034			0.1194
13	0.1972	0.0403		8.9442	0.2666	0.1108	0.0962				0.0477
14	0.1785	0.0074	0.0605	7.1763	0.2291	0.0068		0.0514	0.0619	1.2665	
15	0.1602	0.0101	0.0617	6.236	0.2423	0.0265		0.1807		0.2232	
17		0.0635	0.0577	29.8479	0.2595	0.0761					0.1266
18	0.1874	0.0095	0.0503	5.4834	0.1574	0.0294	0.239			2.1163	0.6581

[a]Metabolite list includes: 27) proline, 28) pyruvate, 29) tyrosine, 30) urea, 31) valine, 32) 3-methylhistidine, 2-aminobutyrate, 33) pyroglutamate, 34) sarcosine, 35) serine, 36) threonine and 37) tyrosine.

Table C4

List of Metabolites Concentrations from Curcumin Supplementation Search and Clear Study

Session 2[a] Time/Suppl.	Metabolite Concentrations in mM Metabolites[b] Curcumin Supplementation Study Session 2												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1/1[c]	0.0166		0.0442	0.0815	0.0781	0.013	0.5474	0.07	0.0891	0.0589	0.0191	0.1156	0.0339
1/1	0.0146	0.105			0.0109	0.0526	0.4889	0.0853	0.0967	0.0543	0.0246	0.1189	0.0267
1/1	0.0101	0.0415	0.070	0.0673	0.0282	0.0092	0.3128	0.0532	0.0761	0.0368	0.0119	0.1023	0.0196
1/1	0.0146	0.1789	0.0416	0.0873	0.0053	0.1145	0.2909		0.1188		0.0287	0.0242	0.027
1/1	0.011	0.1017	0.0373		0.0451	0.0149	0.3598	0.083	0.1314	0.0738	0.0225	0.1209	0.0245
1/2	0.0166	0.0633		0.076	0.0444	0.0386	0.3441	0.0765	0.0799	0.0520	0.0175	0.1109	0.0295
1/2	0.0172	0.0393	0.0488	0.0731	0.0622	0.014	0.5043	0.0641	0.2337		0.0156	0.1225	0.022
2/1	0.0190	0.1998	0.0912	0.1741		0.0655	0.3024	0.0784	0.1098	0.0644	0.0094	0.0835	

2/1		0.0941	0.1065	0.0725	0.0134	0.0148	0.4699	0.0753	0.0886	0.046	0.0152	0.1195	0.0285
2/1	0.0159	0.1220	0.0100		0.0336	0.003	0.4757		0.1195		0.0143	0.1586	0.0288
2/1	0.0133	0.979	0.0183	0.0784	0.0397	0.0142	0.4624	0.0664	0.088	0.0353	0.0206	0.1273	0.0183
2/2	0.0160			0.0804	0.0696	0.0316	0.5543	0.0513	0.089	0.0622	0.0161	0.1213	0.027
2/2	0.0188		0.0224		0.0415		0.5132		0.0667		0.0115		
3/1	0.0153	0.1336		0.0854	0.0425	0.0343	0.5268	0.0793	0.0944	0.0589	0.0184	0.1129	0.0346
3/1	0.0122	0.1648	0.2803	0.0609	0.0748	0.0766	0.3444		0.1509	0.0551		0.1345	0.0349
3/1	0.0129	0.114		0.0816	0.0428	0.0155	0.4026	0.068	0.0849	0.0351	0.0196	0.1145	0.0252
3/2	0.0133		0.0481	0.081	0.0624	0.0141	0.4423	0.0868	0.1622		0.021	0.1159	0.0262
3/2		0.0888	0.0343	0.0279	0.0164	0.0046	0.5171	0.0711	0.3134	0.1018	0.0202	0.1489	0.024
3/2	0.01663	0.1554	0.0481		0.0295	0.0102	0.4551		0.1203	0.0753	0.0179	0.1069	0.016
Metabolites #-Session 3													
1/1	0.0160	0.0936	0.0536		0.0392	0.0248	0.3091	0.078	0.095	0.0625	0.0208	0.1198	0.0253
1/1	0.0152	0.0825	0.0492	0.0852	0.0654	0.0083	0.5382	0.087	0.2475	0.1212	0.0087	0.1358	
1/1	0.0201			0.09512	0.0276	0.3457		0.0134	0.027	0.0158	4.9291		0.1147

Table C4 (continued)

1/2	0.0286	0.0554		0.1065	0.0195	0.0164	0.5776	0.1049	0.0927	0.0692	0.0218	0.1275	0.0325
1/2	0.0129	0.0827	0.0328	0.074	0.0274	0.0104	0.4955	0.057	0.1294		0.0174	0.1211	0.0234
1/2	0.0177	0.2269	0.4701		0.0581	0.089	0.3597	0.0647	0.1594	0.058	0.0116	0.1355	0.0346
1/2			0.0499	0.0393	0.0085		0.4714		0.0669		0.1239	0.0268	0.0231
2/1	0.0156	0.1134		0.0897	0.0594	0.0211	0.582	0.071	0.1171	0.0522	0.0336	0.143	0.026
2/1	0.0151				0.0122	0.003	0.5222		0.1933		0.0131	0.151	0.0269
2/1	0.0163	0.0709	0.0702	0.1012	0.0525	0.0168	0.3639	0.1099	0.1489	0.0553	0.0292	0.1268	0.0276
2/1				0.0503	0.0113	0.0027	0.5913		0.1416		0.0215	0.12	0.0211
2/2	0.0138		0.3850		0.0188	0.0521	0.4909	0.0926	0.0968	0.0441	0.0156	0.1026	0.0201
2/2	0.0221	0.0906		0.0574	0.0478	0.0053	0.3551		0.0691	0.0529	0.0172	0.0857	0.0356
2/2	0.0243	0.1006	0.0962		0.0694	0.0111	0.4572	0.0884	0.0707	0.0507	0.0175	0.1111	0.0419
2/2	0.0158	0.2172	0.4455	0.0522	0.0632	0.0812	0.2852	0.0682	0.146	0.0668	0.0136	0.1369	0.0306
2/2	0.0127	0.0984		0.0739	0.0331	0.0058	0.5846	0.088	0.0833	0.042	0.0289	0.1247	0.0349
3/1	0.0207	0.1156	0.2042	0.092	0.0374	0.0193	0.4834	0.0753	0.0995	0.0744	0.0198	0.1423	0.0376

3/1			0.0992	0.3002	0.0132	0.0181	0.5093		0.1856	0.0274	0.0141	0.1245	
3/1	0.0125		0.3669		0.0113		0.4532		0.0586	0.0295	0.1085	0.0184	0.0266
3/2	0.0386	0.1017		0.1003	0.0151	0.0179	0.5493	0.0768	0.1162		0.0126	0.1501	0.0323
3/2	0.0155	0.1096	0.2448		0.0097	0.0511	0.4317	0.0911	0.0839		0.0167	0.0989	0.0205
3/2		0.1426	0.2042		0.089	0.0724	0.3145		0.1401	0.054	0.0166	0.1144	
3/2	0.0144	0.09	0.0246	0.0837	0.0394	0.0122	0.5914	0.0789	0.0999	0.0354	0.0204	0.1218	

[a]Time of sampling.
[b]Metabolite list includes: 1) pre-exercise, 2) post-exercise, and 3) 30 min. [b]Metabolites included 1) 2-Hydroxyisovalerate, 2) 2-Hydroxyvalerate, 3) 3-Hydroxybutyrate, 4) 4-aminobutyrate, 5)acetate, 6) acetone, 7) alanine, 8) asparagine, 9) betaine, 10) carnitine, 11) creatine, 12) creatinine, and 13) formate.
[c] Supplement was 1) curcumin and 2) placebo.

Table C5

List of Metabolites Concentrations from Curcumin Supplementation Search and Clear Study

Session 2[a] Time/Suppl.	Metabolite Concentrations in mM Metabolites[b] Curcumin Supplementation Study Session 2												
	14	15	16	17	18	19	20	21	22	23	24	25	26
1/1[c]	4.9011	0.186	0.463	1.5808	0.1263	0.0839	0.0898	5.2899	0.1736	0.1934	0.0873	0.0195	0.0036
1/1	4.7784	0.1548	0.6686	1.44	0.1109		0.1214	1.6975	0.2005	0.2001	0.12	0.0489	0.0048
1/1	3.4136	0.0773	0.4081	1.6365	0.1116	0.0732	0.0745	1.0003	0.1371	0.1738	0.0554	0.0169	
1/1	4.6243	0.0831	0.5051	2.0231	0.1537	0.1041	0.0503		0.1583	0.1846	0.7542	0.0211	0.0052
1/1	2.0334	0.1125	0.4187	2.1113	0.1488	0.1008	0.0759	1.512	0.1792	0.2181	0.0513	0.034	0.0039
1/2	4.5083	0.1286	0.4833	1.1436	0.0801		0.0785	1.1773	0.1667	0.1717	0.1242	0.0204	0.0033
1/2	2.0739	0.0993	0.4873	3.2486	0.2213	0.0867	0.1882	5.0372	0.0977	0.1882	0.0871	0.0383	0.0052
2/1	3.5550		0.7138	1.0208	0.6321	0.0922	0.1302	0.925	0.2453	0.1928		0.0614	

2/1	4.3657	0.0898	0.4658	1.4218	0.1054		0.1961	3.7995	0.1453	0.1885	0.0676	0.0156	0.0046
2/1	5.6686	0.1565	0.3944	1.8322	0.1229	0.0868		12.1811	0.1285		0.0993		0.0036
2/1	4.7490	0.1442	0.4019	1.7436	0.1233	0.0955	0.0751	2.6363	0.168	0.2949	0.0945	0.0181	0.0041
2/2	4.6408	0.0777	0.4105	1.1727	0.0978	0.0861	0.0592	6.5419	0.1299	0.1774	0.0845	0.0255	0.0047
2/2	5.0591	0.0791	0.4387	2.0264	0.1122	0.1108		6.1727	0.0891	0.2679	0.0749		0.0058
3/1	4.6761	0.1451	0.5768	1.4495	0.1251	0.0954	0.0753	2.0598	0.1523	0.2260		0.0222	0.0049
3/1	2.1440	0.1307	0.3839	2.7111	0.1947	0.0767	0.0487	2.5245	0.1363	0.2110	0.1057	0.0245	
3/1	4.3464		0.3768	2.2664	0.1519	0.0944	0.0808	1.6435	0.156	0.2994	0.0977	0.0165	0.0035
3/2	2.2793	0.1773	0.6043	2.102	0.1259	0.1399	0.0525	1.6646	0.1468	0.3120	0.1022	0.0126	0.0041
3/2	2.2511	0.2003	0.5329	2.904	0.2205	0.0882	0.0568	2.4838	0.1421		0.0767		0.0084
3/2	1.9648		0.5741	1.708	0.0951		0.0898	5.2899	0.1736	0.1934	0.0873	0.0195	0.0036
Metabolites #-Session 3													
1/1	4.1618	0.1591	0.5039	1.3008	0.1074	0.0968	0.0352	2.475	0.1259	0.1731	0.074	0.0426	
1/1	2.1399		0.4732	2.0163	0.1447	0.0859	0.0911	1.0391	0.1841	0.2343	0.059	0.0175	0.0038
1/1	0.0842	0.0264	1.8547	0.109			0.0972	5.3742	0.1499	0.2005	0.0819	0.0482	0.0061

Table C5 (continued)

1/2	5.3049		0.4305	1.2186	0.1047	0.0865	0.0065				0.0786	0.1261	
1/2	1.7413	0.1536	0.5489	1.7705	0.1081	0.1058	0.0957	6.0407	0.1951	0.1999	0.105	0.0521	0.0042
1/2	2.1991	0.1304	0.4766	2.0861	0.1559	0.0995	0.0547	1.0508	0.1674	0.2529	0.0669	0.0229	0.0022
1/2	4.453	0.0393	0.5894	1.9948	0.1395		0.1086	1.6074	0.1766	0.1924	0.1311	0.0412	0.0042
2/1	5.6915	0.1424	0.4195	1.3779	0.1159	0.1092	0.0963	1.3722	0.1243		0.0845		0.0501
2/1	4.9851	0.1532	0.4207	2.631	0.1597	0.0794	0.0988	11.5683	0.1814	0.2231	0.0453	0.0243	0.0046
2/1	2.2992	0.1709	0.6542	2.0575	0.1481	0.0815	0.0536	12.2675	0.1149	0.1992			0.0062
2/1	2.5207		0.5231	2.2724	0.2798	0.0637	0.0922	1.2682	0.2202	0.2685	0.0986	0.0208	0.0042
2/2	5.2932	0.1446	0.4809	1.3372	0.1011	0.0822	0.1034	5.9544	0.1319	0.2034	0.0654	0.0211	0.0038
2/2	4.3686	0.1138	0.5083	1.4067	0.1165	0.0761		6.322	0.1248		0.0944		0.0046
2/2	3.9292	0.1227	0.4436	1.1817	0.1022	0.075	0.1105	1.0219	0.2013	0.1870	0.0845	0.0177	0.0042
2/2	2.029	0.1491	0.5246	2.9627	0.2161	0.0945	0.0894	1.9063	0.153	0.2192		0.0182	0.0034
2/2	3.4077	0.1744	0.4265	0.948	0.0784	0.0752	0.1373	1.181	0.2173		0.1443	0.0378	0.0037
3/1	5.1268		0.5766	1.1941	0.1112	0.1033	0.0833	2.3598	0.1495	0.2302	0.057	0.0168	0.0038

Table C5 (continued)

3/1	4.3308	0.0172	0.4042	2.6034	0.1706	0.0705	0.0975	1.7921	0.1932	0.1619	0.0882	0.0259	
3/1	4.601	0.0916	0.4341	2.2026	0.1113			6.5446	0.0938		0.0943	0.0133	0.0044
3/2	5.6495	0.0133	0.4649	1.6908	0.1522	0.0827	0.0416	1.5316	0.1022	0.1127		0.0214	
3/2	4.5063		0.5575	0.991	0.0815		0.1188	5.4008	0.175	0.1570	0.0832	0.0196	0.0039
3/2	1.9861	0.1895	0.4061	2.2757	0.1675	0.0757	0.116	2.195	0.1662	0.2598		0.0237	0.0039
3/2	5.1784	0.1917	0.3938	1.0205	0.0931	0.0750	0.1064	2.9618	0.1621	0.1764	0.1166	0.0222	

[a]Time of sampling.
[b]Metabolite list includes: 1) pre-exercise, 2) post-exercise, and 3) 30 min. [b]Metabolites included 14) glucose 15) glutamate, 16) glutamine, 17) glycerol, 18) glycine, 19) histidine, 20) isoleucine, 21) lactate, 22) leucine, 23) lysine, 24) mannose, 25) methionine, and 26) N,N-Dimethylglycine.
[c] Supplement was 1) curcumin and 2) placebo.

Table C6

List of Metabolites Concentrations from Curcumin Supplementation Search and Clear Study

Session 2[a] Time/Suppl.	Metabolite Concentrations in mM Metabolites[b] Curcumin Supplementation Study Session 2												
	27	28	29	30	31	32	33	34					
1/1[c]	0.1785		0.0121		0.0612	3.9886	0.1271	0.005					
1/1	0.192		0.0091	0.1004	0.0651	6.0877	0.2184	0.1222					
1/1	0.1073	0.0473		0.0507		3.8155	0.1924	0.0059					
1/1	0.1475		0.0303	0.0455	0.0511	2.7331	0.2221	0.0118					
1/1	0.1632	0.0585	0.0093	0.09	0.0548	5.4504	0.2673	0.086					
1/2	0.1735	0.0448	0.008	0.0959	0.0379	5.6395	0.1632	0.0937					
1/2	0.1671	0.0961	0.227	0.0466	0.0534	4.1865	0.0894						
2/1		0.0584	0.0255	0.1326	0.0494	4.6985	0.1417	0.0827					

2/1		0.1261	0.194	0.0932	0.0411	3.9621	0.0754	0.089
2/1	0.1302	0.0821	0.0324	0.0971	0.0491	2.3807	0.1272	0.0079
2/1	0.1393	0.0229	0.0162	0.0626	0.0482	5.6001	0.1536	0.0876
2/2		0.0412	0.2813	0.0912	0.0415	5.08	0.1473	0.0873
2/2	0.1772		0.2852	0.1034	0.0425	3.5156		
3/1	0.1686		0.0092	0.0894		5.8336	0.1759	
3/1	0.1129	0.0783	0.0112	0.1105	0.0229	2.3181	0.1586	0.0112
3/1	0.1942		0.0144	0.0728	0.0529	5.476	0.2512	0.0823
3/2	0.193	0.0503	0.0075	0.1391	0.0583	6.1584	0.0852	0.0972
3/2	0.1897	0.0703	0.0124		0.0602	3.8568	0.1693	0.0077
3/2	0.2069			0.1197		3.5859	0.0724	0.0755
Metabolites #-Session 3								
1/1	0.2168	0.0903	0.0073	0.0845	0.0434	6.0048	0.2939	0.0239
1/1	0.1828	0.0624	0.0121	0.1218	0.0608	3.9846	0.078	0.0121
1/1								

Table C6 (continued)

1/2			0.0146		0.0664	5.3258	0.1258	0.0085
1/2	0.2472			0.0953	0.0215	5.286	0.0956	0.0054
1/2	0.1298	0.2161	0.0261	0.0316	0.0455	5.7666	0.2966	0.0133
1/2	0.1723		0.0814			2.4896		0.0122
2/1	0.1649		0.4247	0.0422	0.0481	5.9683	0.274	0.0143
2/1	0.0921		0.0198		0.0407	2.5883	0.0751	
2/1	0.2216	0.0435	0.0082	0.1338	0.0647	5.4451	0.1657	0.0979
2/1	0.2977		0.0136	0.0823		1.9091		0.012
2/2	0.1298		0.0221	0.1458	0.0384	4.7557	0.1911	0.0074
2/2	0.1832		0.0116	0.1095	0.0571	5.3402	0.2897	0.0127
2/2	0.1418		0.0078		0.0281	5.1783	0.2678	0.0075
2/2	0.2033	0.2366	0.0403		0.0241	6.0701	0.1161	0.0158
2/2	0.183	0.0391	0.006	0.1076	0.0668	5.1287	0.1528	0.0055
3/1	0.1539		0.0103	0.0514	0.0562	6.3809	0.2201	0.0197

Table C6 (continued)

3/1	0.1419		0.0171	0.0841		2.7716	0.0586	0.0289
3/1	0.1733		0.1055	0.0742		2.3092		
3/2			0.0138	0.1613	0.0615	5.3397	0.0865	0.0085
3/2	0.1939		0.0207	0.0805		4.9457	0.0821	0.0827
3/2		0.1637	0.0888	0.1090		4.7691	0.0808	
3/2	0.2005	0.0461	0.1536	0.0696	0.0679	4.8914	0.1194	

[a]Time of sampling.
[b]Metabolite list includes: 27) proline, 28) pyroglutamate, 29) pyruvate, 30) threonine, 31) tyrosine, 32) urea, 33) valine, and 34) 3-methylhistidine.
[c] Supplement was 1) curcumin and 2) placebo.

Table C7

List of Metabolites Concentrations from Ketone Salt Supplementation and Exercise Study

	Metabolite Concentrations in mM Metabolites[b] Ketone Salt Supplementation Study												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1/1/2[a]		0.1406	0.0392		0.0115	0.0095	0.5252	0.0676	0.1506	0.0811	0.0234	0.1096	0.5689
1/2/2	0.0092	0.0487	0.0466	0.0843	0.0492	0.0111	0.4947	0.0667	0.1917	0.1168	0.0203	0.1077	0.9888
1/3/2		0.0508	0.0431	0.0704	0.0127	0.0049	0.4894	0.0544	0.1857		0.0185	0.1097	0.9164
1/4/2	0.0083	0.0673	0.0356	0.0587	0.0421	0.01	0.5404	0.073	0.1807	0.1022	0.027	0.1069	
1/1/1	0.0101	0.0894	0.065	0.0553	0.0365	0.0126	0.3824	0.071	0.1965	0.1174	0.0198	0.1037	
1/2/1	0.0099	0.0422	0.1103	0.0658	0.062	0.0475	0.3852	0.054	0.2638		0.0202	0.1015	1.0982
1/3/1	0.0122	0.0684			0.0612	0.0344	0.4295	0.1019	0.2591	0.1106	0.0216	0.1125	0.8060
1/4/1		0.0831	0.0375	0.0769	0.0405	0.0141	0.449		0.1165	0.039	0.0332	0.1125	

Table C7 (continued)

2/1/1	0.0216	0.0588			0.0661	0.0244	0.3915	0.088	0.1217	0.0529	0.0182	0.085	
2/2/1	0.0171	0.108	0.1401		0.0759	0.0477	0.4201	0.0659	0.2523	0.0967	0.0207	0.0888	1.1210
2/3/1	0.0161		0.0678		0.0118	0.003	0.4116	0.1068	0.1825	0.0916	0.0183	0.0916	1.0551
2/4/1	0.0174	0.0948	0.039	0.0593	0.06	0.007	0.4907	0.1003	0.1295	0.0558	0.0236	0.1006	
2/1/2	0.02	0.0448	0.0521	0.0694	0.0498	0.0132	0.5017	0.0844	0.1074	0.0473	0.0117	0.1258	
2/2/2	0.0223	0.0403	0.0412	0.0802	0.0544	0.0163	0.5551	0.091	0.2084	0.122	0.0219	0.0927	1.2074
2/3/2	0.0221		0.0432	0.0704	0.0393	0.014	0.4707	0.079	0.096	0.0499	0.0175	0.0814	1.0009
2/4/2	0.0218	0.059	0.0419	0.0653	0.0454	0.0085	0.4586	0.0826	0.1191	0.0514	0.0209	0.0815	
3/1/1	0.0107	0.1003	0.2701	0.0655	0.047	0.0436	0.2932	0.093	0.1116	0.042	0.0179	0.0881	
3/2/1	0.0106	0.0634	0.1332	0.0712	0.0847	0.0784	0.3877	0.0658	0.1588	0.0411	0.0144	0.097	0.9216
3/3/1	0.0111	0.0909	0.1235	0.0813	0.0729	0.0677	0.3233	0.0706	0.1399	0.0364	0.0139	0.1028	
3/4/1	0.0095	0.0728		0.1062	0.043	0.0137	0.3513	0.0881	0.1538	0.0816	0.012	0.0909	
3/1/2	0.0081	0.0663		0.0934	0.0411	0.0102	0.4348	0.0985	0.1422	0.0777	0.0173	0.0931	0.3822
3/3/2	0.0089	0.0551	0.0337	0.9974	0.0562	0.0119	0.493	0.0913	0.1862	0.1051	0.0159	0.1064	

Table C7 (continued)

3/4/2		0.1713	0.0669		0.0398	0.0167	0.4025	0.105	0.1363		0.0083	0.091	0.4445
4/1/2	0.0167	0.1285			0.0611	0.0098	0.466	0.0844	0.2005	0.1047	0.0188	0.084	
4/1/2	0.0173	0.146		0.1005	0.0956	0.0176	0.5901	0.089	0.2108	0.1381	0.0169	0.1122	0.808
4/2/2	0.0175	0.1263		0.0852	0.0624	0.0138	0.5298	0.0788	0.1419	0.0801	0.0172	0.1085	0.7929
4/2/2	0.0172	0.0592	0.0130		0.0332	0.0091	0.4317	0.1854	0.1242		0.0162	0.0882	
4/2/1	0.0152		0.0558		0.0137	0.0299	0.3992	0.0704	0.1212	0.045	0.0143	0.0781	0.8994
4/4/1	0.0138	0.0387	0.0612	0.0874	0.0107	0.0115	0.4728	0.0672	0.1267		0.0203	0.0904	
5/1/2	0.0087		0.0692		0.0191	0.0109	0.5301		0.104	0.0509	0.1197	0.024	0.4384
5/2/2	0.0097		0.0512	0.0701	0.0173	0.0486	0.5991		0.1654	0.0433	0.1081	0.0184	0.9441
5/3/2			0.0473		0.0208	0.0398	0.5607		0.1615	0.0452	0.113	0.0134	0.6808
5/4/2	0.0145	0.0902	0.0489		0.0199	0.0091	0.6101	0.074	0.0866	0.057	0.0213	0.0913	
5/1/2	0.0154	0.1011	0.0714		0.0373	0.0144	0.4621	0.1401	0.0933	0.043	0.0235	0.1076	0.3771
5/2/1	0.0156	0.0869	0.0536	0.0923	0.0383	0.0145	0.5332	0.0765	0.0882	0.0454	0.0216	0.1034	
5/3/1	0.0168	0.1035	0.0532		0.0356	0.0231	0.5005	0.0782	0.0885	0.0398	0.0121	0.1068	

Table C7 (continued)

5/4/1	0.0169	0.0808	0.0822		0.0482	0.0099	0.564	0.0688	0.0872	0.0524	0.0228	0.0868	
6/1/2	0.017		0.0580		0.0646	0.0123	0.5059	0.1741	0.1028	0.06	0.0225	0.0917	
6/2/2	0.0198		0.046	0.0848	0.1112	0.0166	0.4643	0.0859	0.1072	0.0526	0.011	0.1011	
6/3/2	0.0292		0.0489	0.0742	0.0582	0.0072	0.498	0.1011	0.1023	0.0532	0.0251	0.1133	
6/4/2	0.0179	0.0943	0.0426		0.0692	0.012	0.4883	0.0857	0.096	0.051	0.0125	0.1091	
6/1/1	0.012		0.0524		0.0513	0.0449	0.5003	0.0693	0.1537	0.0493	0.018	0.1017	0.8499
6/2/1	0.0124	0.0414			0.0571	0.0382	0.509	0.0682	0.1483	0.0555	0.0218	0.1072	
6/3/1	0.0139	0.085	0.0512	0.1083	0.104	0.025	0.3194	0.0839	0.0951	0.0576	0.0199	0.0972	
6/4/1					0.055	0.0058	0.3518	0.0775	0.11	0.0527	0.0169	0.1105	
8/1/1				0.075	0.013	0.0415	0.4539		0.1753	0.055	0.0135	0.0753	0.8336
8/2/1	0.0075	0.0737		0.0982	0.0387	0.0112	0.304	0.0694	0.0898	0.0537	0.0182	0.0983	
8/3/1		0.0883		0.1423	0.0118	0.0049	0.3767	0.0753	0.1093	0.0426	0.022	0.102	0.4049
8/4/1		0.0558	0.0487	0.0801	0.0127	0.0038	0.4556	0.0852	0.1073	0.0506	0.0206	0.114	1.0027
8/4/2	0.0102	0.0603	0.0411	0.0935	0.046	0.0131	0.4194	0.0651	0.1076	0.0498	0.0207	0.1097	

Table C7 (continued)

9/1/2	0.0081	0.0656		0.0911	0.0387	0.0116	0.3677	0.0812	0.1009	0.051	0.0188	0.0982	
9/2/2	0.0188	0.1405	0.1473	0.0907	0.1044	0.0187	0.3117	0.0974	0.107	0.0443	0.0146	0.1154	
9/3/2	0.0209	0.1264		0.1088	0.1464	0.0259	0.3754	0.1565	0.1018	0.044	0.0136	0.1364	
9/2/2	0.0193	0.0505	0.1229	0.082	0.0176	0.0182	0.3723	0.0741	0.0943	0.0411	0.0131	0.129	
9/2/1	0.0279				0.0147		0.3909		0.0906				
9/3/1	0.0207	0.1406	0.2022		0.1381	0.0601	0.4201	0.0661	0.1671	0.0477	0.0176	0.1188	
10/1/1	0.0219	0.0822	0.0416		0.079	0.0431	0.4053	0.0609	0.1431	0.0458	0.0161	0.1173	
10/2/1	0.0128	0.1367			0.0637	0.0153	0.48	0.0759	0.1068	0.0616	0.017	0.1127	
10/3/1	0.0122	0.0675	0.0737		0.07	0.0456	0.4347	0.0703	0.1659	0.0548	0.0186	0.1186	0.8602
10/4/1	0.016	0.1202	0.178		0.0964	0.0607	0.5324	0.0722	0.1956	0.0615	0.0196	0.12	
10/1/2	0.011	0.144	0.0505		0.0635	0.0097	0.5123	0.0701	0.1091	0.0597	0.0224	0.1038	0.3564
10/2/2	0.0105	0.0465	0.0394		0.0404	0.0091	0.438	0.0645	0.1086	0.0533	0.0199	0.0901	
10/3/2	0.0304	0.1456	0.1600		0.1005	0.0281	0.9564	0.1214	0.3562	0.2125	0.0278	0.2305	2.6982
10/4/2	0.0088	0.0222	0.042	0.0511	0.0321	0.0092	0.2333	0.0322	0.0879	0.0549	0.0102	0.0565	0.6628

Table C7 (continued)

[a]1/1/2 can be summarized as: Firefighter participant #/time of sampling (pre =1, post-exercise =2, 30 min =3, 24 h =5)/1= ketone salt and 2= placebo.

[b]List of metabolites include 1) 2-hydroxyisovalerate, 2) 2-hydroxyvalerate, 3) β-ydroxybutyrate, 4) 4-aminobutyrate, 5) acetate, 6) acetone, 7) alanine, 8) asparagine, 9) betaine, 10) carnitine, 11) creatine, 12) creatinine, and 13) erythritol.

Table C8

List of Metabolites Concentrations from Ketone Salt Supplementation and Exercise Study

	Metabolite Concentrations in mM Metabolites[b] Ketone Salt Supplementation Study												
	14	15	16	17	18	19	20	21	22	23	24	25	26
1/1/2[a]	0.0531	2.0759		0.6071	2.2154	0.135	0.1043	0.0496	1.1231	0.1542		0.0823	0.0177
1/2/2	0.0374	1.8939	0.0998	0.5027	2.61	0.0642	0.0861	0.0721	1.7817	0.1338	0.159	0.0977	0.0435
1/3/2	0.0515	1.9038	0.0622	0.5168	2.4811	0.0083	0.0888	0.0914	1.6158	0.1218	0.1691	0.0962	0.0399
1/4/2	0.0375	1.6417	0.1322	0.6184	1.4775	0.102	0.0987	0.095	1.2576	0.1729	0.1913	0.1173	0.0352
1/1/1	0.0333	1.9925	0.1257	0.5931	2.4878	0.1815		0.0863	0.8799	0.2036	0.2359	0.1078	0.039
1/2/1	0.0417	1.9031	0.1723	0.5154	1.7768	0.0611	0.0688		1.2995	0.2555	0.2262	0.1069	0.0424
1/3/1	0.0362	1.9091	0.0869	0.5517	2.6397	0.0164		0.0784	1.5993	0.2329	0.2085	0.0993	0.0459

Table C8 (continued)

1/4/1	0.0231	3.9785	0.0962	0.6276	1.2104	0.0802		0.0971	1.2195	0.1678	0.2277	0.0792	0.022
2/1/1	0.0783	4.8997	0.0834	0.4717	1.3541	0.0205			1.5832	0.2007	0.235	0.0401	0.0201
2/2/1	0.0595	3.3728	0.1176	0.4577	1.3159	0.0185		0.0352	2.0989	0.1776	0.2421	0.0519	0.0443
2/3/1	0.049	3.3875	0.0624	0.4613	1.2715	0.0181			1.9902	0.166	0.2277	0.046	0.0181
2/4/1	0.0544	4.7386	0.0919	0.6609	1.1568	0.078	0.1024		1.4149	0.148	0.1786	0.049	0.0481
2/1/2	0.0308	3.8896	0.0966	0.5883	1.2617	0.0843		0.0985	1.8598	0.1486	0.1318	0.0575	0.0418
2/2/2	0.0502	2.0078		0.4587	2.2487	0.0725	0.083	0.074	1.8467	0.1304	0.1994	0.0489	0.0231
2/3/2	0.0363	3.6705	0.1312	0.5024	1.4932	0.0188		0.0676	1.2177	0.1168	0.1811		0.0233
2/4/2	0.0339	5.8605		0.5514	1.0501	0.0545	0.0891	0.0744	1.9051	0.1294	0.175	0.0361	0.0435
3/1/1	0.0318	4.0801		0.5057	0.981	0.0601	0.0873	0.1	0.8381	0.2791	0.1926	0.0785	0.0272
3/2/1	0.0503	4.3264		0.5344	1.7251	0.0621	0.083		1.9816	0.2304	0.1984	0.062	0.0219
3/3/1	0.0366	3.8735	0.1075	0.5128	1.525	0.0154	0.0888		1.1514	0.2029	0.1741		0.0229
3/4/1	0.038	2.1464	0.126	0.541	2.46	0.1916	0.0918	0.0816	0.6104	0.1509	0.2142		0.0225
3/1/2	0.0344	1.8425		0.5319	2.6927	0.0295		0.0897	1.2436	0.1323		0.0509	0.0224

Table C8 (continued)

3/3/2	0.0296	2.0178		0.4943	2.212	0.1799	0.0929	0.0432	0.7525	0.1539	0.2577		0.0233
3/4/2	0.0314	2.1533	0.1121	0.4703	2.0056	0.1062	0.072	0.0472	0.7489	0.1792	0.2136	0.0693	0.0153
4/1/2	0.0385	2.2566		0.4813	2.8856	0.0123	0.078	0.0505	3.197	0.1809	0.2024	0.0563	0.0186
4/1/2	0.0322	2.0094		0.455	2.5448	0.1427		0.0545	1.5759	0.1631	0.2068	0.057	0.0202
4/2/2	0.0365	1.898	0.1171	0.3934	2.0097	0.1487		0.0941	1.0376	0.152	0.2021	0.1449	0.0182
4/2/2	0.0348	4.5456	0.1078	0.4847	2.2734	0.0789	0.0619		1.1893	0.2134	0.1131		0.0145
4/2/1	0.0325	1.9345	0.1143	0.4696	1.8862	0.124	0.0714	0.0579	1.3672	0.1766	0.13	0.0551	0.0376
4/4/1	0.0291	4.9812	0.1113	0.4541	2.1648	0.1046	0.0581		1.4866	0.1737			
5/1/2	0.0282	5.5002	0.0802	0.5038	1.695	0.2545	0.0493		2.6596	0.2272	0.1471		
5/2/2	0.0212	4.8659	0.0942	0.4112	1.4846	0.2092			2.031	0.1867			
5/3/2	0.0295	4.626	0.0712	0.4874	1.2263	0.1029	0.0873	0.0662	1.9022	0.1728	0.1474	0.0821	0.0224
5/4/2	0.0328	4.7978	0.1073	0.5015	1.3535	0.0942	0.1027	0.1078	1.3215	0.1834	0.2436	0.1181	0.0431
5/1/2	0.028	4.6627		0.5146	1.4413	0.0105		0.1187	2.2125	0.1645	0.243		0.0457
5/2/1	0.0313	4.585	0.1486	0.4974	1.5785	0.0171	0.0992	0.1231	1.7884	0.1678	0.2452	0.1049	0.0237

Table C8 (continued)

5/3/1	0.031	4.9161		0.5506	0.9944	0.0858	0.1067	0.0755	1.4177	0.1841	0.2271		0.0218
5/4/1	0.0301	3.2845	0.1845	0.4771	1.2757	0.0963	0.0979	0.0962	1.5038	0.1856	0.1964		0.0215
6/1/2	0.0441	4.7242		0.4223	1.6047	0.057	0.0891	0.0747	1.4653	0.1629	0.198		0.0214
6/2/2	0.0317	3.9378		0.475	1.1875	0.0097		0.1073	1.2229	0.1592		0.0763	0.0211
6/3/2	0.0304	4.4578	0.1926	0.4877	1.1349	0.0904	0.0909	0.0614	0.9576	0.168			0.0233
6/4/2	0.0267	4.2357		0.4741	0.8426	0.0699		0.0819	1.106	0.1593	0.1936	0.0963	0.0217
6/1/1	0.025	4.2349		0.5228	1.3707	0.0145			1.3877	0.2027	0.201	0.0593	0.0185
6/2/1	0.0294	3.8554	0.1475	0.4854	1.3039	0.0193			1.4354	0.1975	0.2048	0.084	0.0475
6/3/1	0.0222	3.9935	0.2019	0.4734	1.0379	0.0791	0.0882	0.1301	0.7926	0.1764	0.206	0.0997	0.0187
6/4/1	0.0331	4.4114		0.4813	1.1357	0.0722	0.0872	0.0693	0.9352	0.1338		0.0396	0.0222
8/1/1	0.0343	4.3747	0.2174	0.3383	1.5164	0.0092	0.0497		1.9314	0.1766	0.1677		
8/2/1	0.0295	3.9445	0.1463	0.4429	1.5805	0.01	0.0874		1.3108	0.1941	0.1954	0.0819	0.0212
8/3/1	0.0343	3.7753	0.1672	0.4173	1.0742	0.0869	0.0864	0.0858	0.9915	0.1432	0.1909	0.0601	0.0189
8/4/1	0.0300	4.6659	0.1831	0.4945	1.0938		0.1015	0.0862	1.0893	0.1552		0.0725	0.0221

Table C8 (continued)

8/4/2	0.0255	4.0148	0.0244	0.5218	1.396	0.0521	0.0981	0.0853	1.2827	0.1476		0.0673	0.0214
9/1/2	0.038	3.8081		0.5066	1.3949	0.0251		0.0794	1.1087	0.1446	0.1781	0.0469	0.0479
9/2/2	0.0275	4.36	0.1816	0.4591	1.1798	0.0854	0.0902	0.1018	0.8623	0.1471	0.2		0.0449
9/3/2	0.0542	5.1208	0.1709	0.3835	0.9515	0.0433	0.0732	0.0406	1.0903	0.1722	0.1708	0.0738	0.0393
9/2/2	0.0389	4.1958		0.3819	1.4913	0.0124	0.0879	0.0421	1.9898	0.1765	0.2026	0.0764	0.0463
9/2/1	0.0404	3.8036		0.336	1.4823	0.0206	0.0849	0.1032	2.5118	0.1584	0.2119	0.082	0.0212
9/3/1		4.0513	0.1115		1.318	0.2566				0.0846			
10/1/1	0.0376	4.6904	0.1845	0.5096	1.4177	0.0614	0.0772		2.5244	0.2575	0.2593	0.0602	0.0231
10/2/1	0.0278	3.9824	0.1672	0.5039	1.3333	0.0105	0.0695		1.9281	0.2307	0.2077	0.0833	0.0213
10/3/1	0.0272	4.4896	0.0788	0.5149	1.0084	0.0721	0.1005	0.0523	1.3663	0.2137	0.2403	0.0529	0.0222
10/4/1	0.0505	4.4779	0.2886	0.5106	1.1923	0.0197	0.0987		1.4177	0.2378	0.2356	0.0604	0.0207
10/1/2	0.0537	4.8294	0.2902	0.502	1.5874	0.008	0.0999		1.8693	0.2715	0.2853	0.0711	0.0245
10/2/2	0.0299	5.4607		0.5467	0.7512	0.0179	0.1071	0.0446	1.4101	0.1805	0.2877	0.0642	0.0195
10/3/2	0.0362	5.9506	0.0632	0.4236	1.5401	0.0169	0.0977	0.0961	1.4233	0.1694	0.2499	0.0577	0.0173

Table C8 (continued)

10/4/2	0.0562	3.6623	0.9368	4.2075	0.5526	0.1778	3.6763	0.3442	0.6215	0.2652	0.0435

[a]1/1/2 can be summarized as: Firefighter participant #/time of sampling (pre =1, post-exercise =2, 30 min =3, 24 h =5)/1= ketone salt and 2= placebo.
[b]List of metabolites include 14) formate, 15) glucose, 16) glutamate, 17) glutamine, 18) glycerol, 19) glycine, 20) histidine, 21) isoleucine, 22) lactate, 23) leucine, 24) lysine, 25) mannose, and 26) methionine.

Table C9

List of Metabolites Concentrations from Ketone Salt Supplementation and Exercise Study

	Metabolite Concentrations in mM Metabolites[b] Ketone Salt Supplementation Study												
	27	28	29	30	31	32	33	34	35	36	37	38	
1/1/2[a]		0.0328	0.1579	0.0479	0.0089			0.0991	0.0672	4.1751	0.2687	0.0102	
1/2/2		0.0228	0.1589	0.0604	0.1298	0.0045	0.2118	0.0895	0.0512	3.8565	0.1391	0.0068	
1/3/2	0.0024	0.0331	0.1898		0.0081	0.0061	0.1745		0.0565	3.9015	0.0964	0.0057	
1/4/2	0.0025	0.025	0.1918	0.0579	0.0071	0.0028			0.0648	3.4484	0.2374	0.0049	
1/1/1	0.0022	0.0524	0.1993	0.0772	0.0771		0.1886	0.084	0.0765	4.2418	0.2522	0.0797	
1/2/1	0.0025		0.1785		0.022	0.0091		0.0916	0.0581	4.0808	0.0758	0.0049	

Table C9 (continued)

1/3/1		0.0222	0.2176	0.0677	0.0168	0.0056	0.1354		0.0564	4.3736	0.0849	0.0081
1/4/1		0.0416	0.2699	0.0778	0.0638		0.0937		0.0677	4.14	0.0821	0.1001
2/1/1	0.0028	0.0206	0.1897		0.0232	0.0377			0.0226	4.6054	0.0671	0.0859
2/2/1	0.0025		0.1703	0.0485	0.0163	0.0056		0.0651		4.8008	0.0391	0.0064
2/3/1	0.0027	0.014	0.1728	0.0859	0.0233	0.0043	0.2062	0.0807	0.029	4.9825	0.0342	0.0814
2/4/1	0.0029	0.0281	0.2246		0.0108		0.1975	0.1354	0.0668	5.2667	0.1396	0.0051
2/1/2	0.0024		0.2578		0.0097	0.0077	0.478		0.0463	5.3975	0.0853	0.0899
2/2/2	0.0026	0.0493	0.261		0.0149			0.0656	0.0471	4.544	0.1613	0.0107
2/3/2	0.0025		0.206		0.0079	0.0024			0.0401	4.2789	0.1412	0.0862
2/4/2	0.0027		0.1719	0.0595	0.012		0.1718	0.178	0.0485	4.5261	0.0465	
3/1/1	0.0036		0.1511	0.1024	0.0168	0.0284				6.1578	0.0986	0.0077
3/2/1	0.0033	0.0221	0.1735		0.0251	0.0343			0.0437	6.2155	0.0879	0.0082
3/3/1	0.0033		0.1797		0.0158			0.0632		6.1207	0.0758	0.0083

Table C9 (continued)

3/4/1	0.0028		0.2038		0.01	0.0062			0.0519	6.3924	0.2523	0.0098
3/1/2	0.0042		0.1433	0.0199	0.0085	0.0039	0.1307	0.0573	0.064	5.4261	0.1548	0.0078
3/3/2	0.004		0.1485		0.0073	0.0273	0.1554	0.0497	0.0396	5.1921	0.145	0.0055
3/4/2	0.0035	0.0442	0.1533		0.0232		0.1529	0.0613	0.0692	4.247	0.2683	0.009
4/1/2	0.0036	0.0163	0.1477		0.0117		0.1488		0.0658	4.3579	0.2742	0.0075
4/1/2	0.0035		0.1415		0.0098	0.0043	0.1531	0.0586	0.0578	4.3327	0.1454	0.0065
4/2/2			0.1983		0.0717		0.1596	0.0489	0.0571	4.9598	0.2538	0.0683
4/2/2	0.0035		0.1457		0.0136	0.0036	0.1672			3.6424	0.0688	0.0092
4/2/1	0.0037		0.2223		0.0813	0.0076	0.1611	0.1292	0.0684	5.1043	0.1789	0.0045
4/4/1		0.0187	0.1843		0.0162					2.9881		0.0059
5/1/2			0.1729		0.0408			0.0119		3.1123		
5/2/2			0.1854		0.0268	0.0056		0.0216		3.1056		
5/3/2	0.0039		0.2602		0.0124	0.0053			0.0544	3.6622	0.2701	0.0088
5/4/2	0.0034	0.0148	0.2197	0.0347	0.0109		0.1644	0.1279	0.0625	4.0199	0.2162	0.0063

Table C9 (continued)

5/1/2	0.0039	0.0166	0.1903		0.0115	0.0036		0.1322	0.0296	3.823	0.0771	0.0997
5/2/1	0.0038		0.1691	0.0446	0.0203		0.1218	0.1327	0.0482	3.9025	0.0791	0.013
5/3/1	0.0036	0.0472	0.2526		0.0097	0.0096	0.1477	0.1355	0.0748	3.7406	0.2913	0.0047
5/4/1	0.004				0.0859		0.1532	0.1479		3.8682	0.2809	0.0235
6/1/2	0.0039	0.0213	0.1988		0.0099	0.0072	0.1488	0.1428	0.0469	3.967	0.1343	0.0136
6/2/2	0.0049				0.0095	0.0036	0.1529	0.1544	0.0558	3.9404	0.0966	0.1049
6/3/2	0.004		0.2241		0.0134	0.0039		0.15	0.0483	5.2371	0.2451	0.0063
6/4/2	0.003		0.1948		0.0088			0.1773	0.0243	4.137	0.0988	0.08
6/1/1	0.004		0.1589		0.0275	0.0041	0.1841	0.1652	0.0479	4.0827	0.0599	0.0823
6/2/1	0.0033	0.0175	0.15		0.0127	0.0049	0.1774	0.1322	0.0508	4.3365	0.0655	0.0856
6/3/1	0.0035		0.1827	0.0395	0.0108			0.1352	0.0498	5.4848	0.0896	0.0077
6/4/1	0.0028	0.0166	0.1869		0.0102	0.0155	0.1993		0.0429	3.1582	0.1744	0.007
8/1/1			0.0959		0.0334		0.2007			3.2242		0.0057
8/2/1	0.0023		0.2216	0.0606	0.0128			0.1653	0.0398	3.2903	0.0504	0.0049

Table C9 (continued)

8/3/1	0.0021		0.1955		0.0124	0.0105		0.1491	0.0372	4.1962	0.2366	0.0087
8/4/1	0.0032	0.0182	0.1679		0.0153		0.191	0.1722	0.0554	3.493	0.223	0.0152
8/4/2	0.0037	0.018	0.1267	0.074	0.0889		0.2016	0.1349	0.0528	3.6115	0.2103	
9/1/2	0.0025	0.0202	0.1836	0.0556	0.0701	0.0011	0.1802	0.0999	0.0563	3.3594	0.2214	0.0102
9/2/2	0.0026		0.2436		0.0132	0.0107	0.1544	0.1229	0.0527	3.7674	0.239	0.0139
9/3/2	0.0029	0.0432	0.1785	0.0638	0.0743	0.0054	0.1541		0.0578	4.1532	0.1603	0.0168
9/2/2	0.0028		0.1442		0.0145	0.0029	0.1104	0.1326	0.0625	3.8911	0.1777	0.0046
9/2/1	0.0034				0.014	0.0188	0.1302	0.1385	0.0611	3.9401	0.0731	0.0068
9/3/1					0.0648	0.0231	0.1556	0.1563		19.163		
10/1/1	0.0037	0.0226		0.0827	0.0088	0.0076	0.1623		0.0545	4.0602	0.0881	0.0073
10/2/1	0.0035		0.1894	0.0582	0.0105	0.0372	0.1627	0.0922		4.1206	0.077	0.0083
10/3/1	0.0031	0.049	0.2119	0.0381	0.0123	0.0321		0.1127	0.0567	4.8578	0.1669	0.0075
10/4/1	0.004	0.0135	0.2449	0.0658	0.0188		0.1119		0.0437	5.0898	0.0769	0.006
10/1/2	0.0041		0.2068	0.1103	0.0168		0.1945	0.1172	0.0514	5.1943	0.0783	0.0087

Table C9 (continued)

10/2/2	0.0031	0.0529	0.2127	0.0516	0.0088	0.0058	0.1774	0.1041	0.062	4.4928	0.2548	0.0076
10/3/2	0.0037	0.0497	0.2194	0.0412	0.1117				0.0515	4.0339	0.2758	0.0065
10/4/2	0.0069	0.1002	0.42	0.1505	0.0156	0.0099	0.3901	0.2311	0.1124	9.7926	0.5677	0.0141

[a]1/1/2 summarized as: Firefighter participant #, /time of sampling (pre =1, post-exercise =2, 30 min =3, 24 h =5), and /1= ketone salt and 2= placebo.
[b]List of metabolites include 27) N,N-dimethylglycine, 28) phenylalanine, 29) proline, 30) pyroglutamate, 31) pyruvate, 32) sarcosine, 33) serine, 34) threonine, 35) tyrosine, 36) urea, 37) valine, and 38) 3-methylhistidine.