DIFFERENTIAL METABOLITE EXPRESSIONS IN FIREFIGHTERS INDUCED BY FIREGROUND EXPOSURE: A COMPARATIVE METABOLOMICS ANALYSIS

by

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We respectfully acknowledge the University of Arizona is on the land and territories of Indigenous peoples. Today, Arizona is home to 22 federally recognized tribes, with Tucson being home to the O'odham and the Yaqui. Committed to diversity and inclusion, the University strives to build sustainable relationships with sovereign Native Nations and Indigenous communities through education offerings, partnerships, and community service.

Dedication

To my parents and Sherry.

Table of Contents

List of Figures

List of Tables

Abstract

Firefighters are regularly exposed to known or probable carcinogens, including polycyclic aromatic hydrocarbons (PAHs), benzene, formaldehyde, phthalates, and other harmful substances. This exposure occurs mainly through inhalation of smoke released during fire events and dermal exposure. Consequently, firefighters face a higher risk of selected cancers, such as bladder cancer. The International Agency for Research on Cancer (IARC) classified firefighters' occupational exposure as carcinogenic to humans, but there is still a lack of mechanistic evidence on what and how fireground exposure elevates cancer risks and the relationship between fireground exposure and metabolite expression in humans remains poorly understood. Research is also limited regarding the differences between wildland-urban interface (WUI) firefighting and structure fires concerning the biological response in firefighters. Additionally, there is a need to understand how women firefighters respond differently to fireground exposure compared to men firefighters. To address these gaps, we bring together three projects involving male and women firefighters, exposed to various types of fires. Powered by the high-resolution metabolomics pipeline and the high-resolution liquid-chromatography mass-spectrometry (LC-MS) platform, these projects aim to evaluate the impact of fireground exposure on firefighters' metabolisms, together with other important factors for firefighters:

Project 1 assesses changes in the urinary metabolome by Hispanic ethnicity among male firefighters respond to structure fires. Prior to project 1, we developed an analytical pipeline for urine-based metabolomics, which was applied to investigate the effect of fireground exposure (prior analysis) and ethnicity (project 1) on metabolome as disparity in cancer risk has been observed among Hispanic and non-Hispanic firefighters. Two publications have been produced from project 1, titled “Evaluating changes in firefighter urinary metabolomes after structural fires: an untargeted, high-resolution approach” and “Differential metabolic profiles by Hispanic ethnicity among male Tucson firefighters”.

Project 2 assesses changes in the urinary metabolome by fireground exposure in male firefighters responding to WUI fires. WUI fires differ from structure fires in that they introduce a

much more complex exposure matrix due to the involvement of both wildland biomass and built materials. We also tried to compare the metabolic responses across different fire types, looking for unique and shared biological responses that might understand prevalent conditions among these firefighters. One manuscript has been produced based on project 2 which is being peer reviewed as of the time of dissertation process.

Project 3 assesses changes in the urinary metabolome by training fire exposures in women firefighters. Project 3 investigates metabolic responses to training fire exposures among women firefighters, differing from previous projects in both exposure matrix and population. Although training fires are resembling structure fires regarding burning materials, they are intrinsically different in fire intensity and participants activities. We also compared metabolic responses by fire exposure across two populations (genders).

Chapter 1. Introduction

1.1 Background

Firefighters face increased health risks as compared to the general population owing to frequent exposure to hazardous occupational exposures. In 2022 the International Agency for Research on Cancer (IARC) determined that occupational exposure as a firefighter was carcinogenic (Group 1)[1]. Based on their meta-analysis combining studies internationally, IARC found sufficient evidence for two cancers, mesothelioma and bladder cancer, and increased rates and limited evidence for cancers of the colon, prostate and testis, as well as melanoma and non-Hodgkin lymphoma. Furthermore, IARC found “strong” evidence for five key characteristics of carcinogens in relation to occupational exposure as a firefighter, including genotoxicity, epigenetic changes, chronic inflammation, oxidative stress, and receptor-mediated activation.

Firefighters are exposed to multiple known and suspected carcinogens, including but not limited to polycyclic aromatic hydrocarbons (PAHs), benzene, formaldehyde, and asbestos[2]. Multiple studies have evaluated PAHs in urine, and persistent organic pollutants (POPs) in blood. Silicone wristbands devices have been used to measure exposures to semi-volatile organic chemicals (SVOCs) in a wide range of settings including municipal structure firefighters’ occupational exposure using passive sampling[3-6], for measuring exposure to PAHs. In a community-engaged study of municipal firefighters in North Carolina, wristbands were also used to measure exposures to phthalates, brominated flame retardants (BFRs), organophosphate esters (OPEs) and per- and polyfluoroalkyl substances (PFAS), in addition to PAHs[3]. This study reported increased PAHs levels after on-duty fire exposures. Regarding wildland fires, one study reported that wildland firefighters experience high concentrations of VOCs after suppressing wildland fires by various operations[7]. In sum, both municipal and wildland firefighters experienced elevated levels of hazardous exposures after fire exposures. As wildland-urban interface (WUI) fires mix smoke from both structures and wildland fuel materials, the WUI exposure matrix is more complex.

In addition to the higher cancer risks than the general population, partially owing to their adverse exposures, the cancer rate disparity among firefighter subgroups also presents a challenge. A cancer-registry based in California found variability in cancer risk by race and ethnicity, with minority firefighters (approximately 2/3rd Hispanic and 1/3rd African American) evaluated as one group[8]. Among the 32 examined cancers, there were six (tongue, testicular, bladder, non-Hodgkin lymphoma, chronic lymphocytic leukemia, and chronic myeloid leukemia) that were significantly elevated only among the racial/ethnic minority firefighters[8].

Gender seems to play an important role in the observed disparities. Despite increasing studies on the health effects of firefighting, evidence on its impact among women firefighters remains sparse and evidence is mixed. Some studies report that women firefighters had significantly increased bladder (SMR=33.51; 4.06 -121.05, sample size < 5), brain (aOR=2.54; 1.19-5.42), and thyroid (aOR=2.42; 1.56-3.74) cancer risk[9-11]. However, a study on mortality and cancer incidence among Australian women firefighters showed a similar cancer rate compared with the general population[12]. Toxic exposure may contribute not just to cancer but also to reproductive conditions in women firefighters. Previous study show that women firefighters have high rates of adverse reproductive outcomes, including miscarriage[1], preterm birth[1], and lower levels of anti-müllerian hormone (AMH)[2], an important diagnostic measure directly associated with reproductive reserve.

The relationship between structure and wildland fire exposures and associated adverse health outcomes in firefighters remains poorly understood. Additionally, WUI firefighting introduces firefighters to a range of toxicants seen in structure and wildland fires, such as benzene and PAHs, which are known to be carcinogens. However, systematic identification and assessment of WUI fire-related metabolites and metabolic changes is lacking. Furthermore, the relationship between fireground exposure and adverse health effects in women firefighters is not well understood. To address these gaps, we performed urine metabolomics analyses which enabled a comprehensive evaluation of the changes induced by fireground exposures, while controlling other variables, to glean systematic alteration in metabolism. Moreover, they will facilitate the identification of biomarkers for environmental contaminants and diseases of interest, providing insights into the exposure-disease relationship and effective preventions, such as bladder cancer and informing the development of effective interventions.

In sum, the links between toxic exposure and cancer risks are of paramount importance in ensuring the health and well-being of firefighters, a group often overlooked in research. Existing evidence lacks a comprehensive understanding of the relationship between fireground exposure and adverse health effects. By focusing on this understudied population, this thesis aims to fill the significant knowledge gap. Given the increasing recognition of occupational health and safety, this research is also poised to make a significant contribution to the occupational well-being of firefighters. The findings of this study could influence policy decisions, training protocols, and health assessments for firefighters and other professionals working in similar high-risk environments. This multifaceted study encompasses a range of data sources, including men and women firefighter datasets, which covers exposure scenarios of wildland fires, structure fires, and training fires that provides valuable insights into potential variations based on gender and fire type. Evaluation of metabolic changes by fire exposure in various fireground scenes, together with comparisons between firefighter subgroups, will not only facilitate urinary biomarker discovery that can serve as non-invasive indicators of environmental contaminants and prevalent cancers in firefighters, but also assist in improving our mechanistic comprehension of the exposure-disease relationship that is crucial in formulating effective preventive actions.

1.2 Related work

Firefighters face significant occupational exposure to hazardous chemicals, including PFAS, PAHs, benzene, and wildfire smoke toxicants. These exposures occur through multiple pathways, such as inhalation, ingestion, and dermal absorption, and contribute to increased cancer risks and other health concerns. In this section, we present a concise review of related works on firefighters' occupational exposure, health risks as compared to the general population, and other omics studies on firefighters.

1.2.1 Firefighters' occupational exposures

Firefighters suppressing structure fires are frequently exposed to PAHs and benzene, both of which are classified as carcinogenic. These chemicals are generated during the combustion of building materials, plastics, and other synthetic substances. PAHs and benzene exposure occur through inhalation and dermal absorption, with studies detecting these chemicals in firefighters' air samples, exhaled breath, urine, and skin wipes. Even when wearing full protective gear, PAHs can penetrate turnout gear and accumulate on the skin, particularly around the neck where protection is limited. Additionally, the process of removing contaminated gear can lead to further exposure as PAHs and benzene off-gas from fire-damaged materials[13].

One of the major sources of occupational exposure for firefighters is PFAS, a class of synthetic chemicals used in turnout gear, aqueous film-forming foam (AFFF), and fire station dust. PFAS compounds are highly persistent in the environment and bioaccumulate in the human body, leading to elevated levels in firefighters' blood serum. Studies have linked PFAS exposure to increased risks of testicular, kidney, and prostate cancer, with firefighters exhibiting significantly higher levels of these chemicals compared to the general population[14].

Wildland firefighters are at high risk of exposure to toxicants from wildfire smoke, which contains a complex multipollutant mixture of hazardous chemicals. Unlike firefighters responding to structure fires, wildland firefighters often lack adequate respiratory protection, as self-contained breathing apparatuses (SCBA) are impractical for prolonged deployments. Long-term exposure to wildfire smoke has been associated with a 43% increased risk of lung cancer and a 30% increased risk of cardiovascular disease among career wildland firefighters responding to WUI fires[15].

Firefighters face significant occupational exposure to a wide range of hazardous chemicals, which are linked to increased risks of cancer and other chronic health conditions. Studies using silicone wristbands and dog tags as passive sampling devices have quantified these exposures, revealing that firefighters are exposed to complex mixtures of chemicals, including PAHs, flame retardants (both brominated and organophosphate), phthalates, and PFAS[3, 6]. These exposures are particularly elevated during firefighting activities, such as responding to structure fires, where burning materials release toxic compounds into the air. For example, PAH concentrations were found to be up to 8.5 times higher during fire responses compared to off-duty periods, with higher molecular weight PAHs like pyrene and fluoranthene being particularly prevalent[3, 6]. Additionally, PFAS, particularly PFOS, were detected at higher levels during firefighting activities, likely due to their presence in firefighting gear and fire suppression foams[3].

The type and extent of chemical exposure can vary depending on the fire scenario, the materials burning, and the firefighter's role during the response. Firefighters involved in active fire suppression or overhaul (searching for and extinguishing smoldering materials) tend to have higher levels of chemical contamination on their personal protective equipment (PPE) compared to those in less exposed roles, such as incident commanders or pump operators. Gloves have been found to be more contaminated than jackets, suggesting that handling burned materials or crawling on contaminated surfaces contributes significantly to dermal exposure. Routine gross decontamination of PPE, such as washing with soap and water, has been shown to reduce some contaminants, but its effectiveness varies by chemical class. For instance, while brominated flame retardants like BDE-209 are effectively removed, organophosphate flame retardants like TDCPP may persist or even increase due to potential cross-contamination during decontamination[16].

1.2.2 Firefighters' health risks

Firefighters face significant health risks due to their exposure to fire-related hazards, including smoke, toxic chemicals, and extreme physical demands. These exposures can lead to both acute and chronic health conditions, particularly cardiovascular, respiratory, and cancer risks. This section summarizes studies reporting health risks observed among firefighters.

Firefighters are at risk of systemic inflammation and oxidative stress due to their exposure to smoke and other toxic substances. Hejl et al. (2012) found that wildland firefighters exposed to wood smoke during prescribed burns had significantly increased levels of interleukin-

8 (IL-8), a marker of systemic inflammation. This inflammatory response can contribute to the development of chronic diseases, including cardiovascular and respiratory conditions[17].

Exposure to smoke and particulate matter (PM) at fire scene poses significant respiratory risks. Ferguson et al. (2016) conducted a controlled human exposure study and found that firefighters exposed to wood smoke exhibited increased levels of inflammatory biomarkers such as IL-8 and pentraxin-3, indicating systemic inflammation. This inflammation can lead to acute respiratory distress and long-term respiratory conditions. The study also noted that firefighters who performed tasks involving direct exposure to smoke, such as lighting fires, had the highest increases in IL-8 levels, suggesting a dose-response relationship between exposure and inflammation[18].

Firefighters are at an increased risk of cardiovascular diseases due to the strenuous nature of their work and exposure to smoke and chemicals. A study by Byczek et al. (2004) found that firefighters have a higher prevalence of obesity, elevated cholesterol, and hypertension compared to the general population. These factors contribute to a higher risk of coronary heart disease. The study also highlighted that elevated body mass index (BMI) in firefighters is significantly associated with higher blood pressure, triglycerides, and glucose levels, further exacerbating cardiovascular risks[19].

Firefighters are at an elevated risk for several types of cancer due to their exposure to carcinogens present in smoke and burning materials. Firefighters have shown elevated risks for cancers of the digestive system, particularly esophageal, colorectal, and stomach cancers. Daniels et al. (2014) reported a significant increase in esophageal cancer (SMR=1.39) and colorectal cancer (SMR=1.30) among firefighters. Similarly, Tsai et al. (2015) found that firefighters had a 1.6-fold increased risk of esophageal cancer, particularly adenocarcinoma, which may be linked to the inhalation of smoke and dust during fire suppression activities. Lung cancer is another significant concern, with studies indicating a modest increase in risk. Daniels et al. (2014) observed a 10% increase in lung cancer mortality (SMR=1.10) among firefighters. Tsai et al. (2015) also noted an elevated risk for non-specific non-small cell lung cancer among firefighters, although the overall risk for lung cancer subtypes was not consistently significant.

Firefighters are at an increased risk for cancers of the prostate, bladder, and kidneys. Daniels et al. (2014) reported a 29% increase in kidney cancer mortality (SMR=1.29), while Tsai et al. (2015) found a 27% increased risk of kidney cancer among firefighters. Prostate cancer was also significantly elevated, with a 26% increased risk observed in the meta-analysis by Soteriades et al. (2019). Firefighters have shown elevated risks for certain hematologic cancers, including non-Hodgkin lymphoma (NHL), multiple myeloma, and leukemia. Daniels et al. (2014) reported a 17% increase in NHL mortality (SMR=1.17), while Tsai et al. (2015) found a significant increase in acute myeloid leukemia (AML) among firefighters, particularly in white firefighters. Melanoma risk is notably higher among firefighters. Tsai et al. (2015) reported a 1.8-fold increased risk of melanoma, which may be linked to exposure to PAHs and other carcinogens during firefighting activities. Soteriades et al. (2019) also found a consistent increase in melanoma risk across multiple studies. Firefighters have shown an increased risk of brain cancer, with Daniels et al. (2014) reporting a 10% increase in brain cancer mortality (SMR=1.10). Tsai et al. (2015) also found a significant elevation in brain cancer risk among firefighters, particularly in non-white firefighters.

1.2.3 Omics studies

This section reviews three key studies that utilize omics techniques to investigate the health risks associated with firefighting, with a focus on epigenetic and metabolic changes that may contribute to increased cancer risk and other adverse health outcomes. These studies collectively highlight the potential of omics approaches to uncover biological mechanisms underlying the elevated health risks faced by firefighters and provide insights into the development of biomarkers for early detection and prevention.

Goodrich et al. (2025) examined longitudinal changes in miRNA expression and DNA methylation in firefighters exposed to WUI fires. Blood samples from 99 firefighters were collected before and after a wildfire season, and miRNA expression and DNA methylation were analyzed using the nCounter Human v3 miRNA expression panel and the Infinium EPIC array, respectively. The study found significant changes in miRNA expression, with 65 miRNAs differentially expressed at follow-up at 10 months compared to baseline. Notably, miR-518c-3p, a tumor suppressor, was downregulated in firefighters who responded to WUI fires. However, no significant changes in DNA methylation were observed. These findings suggest that WUI fire exposures alter miRNA expression within a 10-month time span, potentially linking these changes to increased cancer risk, while DNA methylation remains relatively stable over this time. This study underscores the importance of miRNA as a sensitive biomarker for acute exposures and highlights the need for further research into the long-term epigenetic effects of WUI firefighting[20].

Goodrich et al. (2022) examined whether DNA methylation, an epigenetic marker, changes over time in firefighters and whether such changes are associated with occupational exposures. Researchers followed 50 non-smoking new recruits from the Tucson Fire Department, collecting blood samples before live fire training and again 20–37 months later. Using the Infinium MethylationEPIC array, they identified 680 CpG sites with significant methylation changes, 60 of which showed at least a 5% difference. Many of these sites were in genes involved in cancer, neurological function, and cell signaling. Notably, 140 of the CpG sites were associated with cumulative fireground exposures (fire-hours and fire-runs), with structure fire-runs showing stronger associations than total fire exposure. These changes appeared linked to chronic rather than recent exposures, suggesting a lasting epigenetic impact of firefighting. The study provides early evidence that even short-term service in firefighting can alter DNA methylation, supporting its potential use as a biomarker for cumulative exposure and future disease risk, particularly for cancer and cardiovascular conditions[21].

Mrowiec et al. (2023) analyzed serum metabolome profiles in pre-diagnostic samples from women who developed breast cancer during a 15-year follow-up in the HUNT2 study. Serum samples from 453 case-control pairs were analyzed using high-resolution mass spectrometry to quantify 284 metabolites. The study found that age was a major confounding factor. Increased levels of glycerides, phosphatidylcholines, and sphingolipids were associated with reduced breast cancer risk in younger and middle-aged women, while increased lipid levels were linked to higher risk in older women. The study concluded that changes in metabolite levels, reflecting impaired lipid and amino acid metabolism, are associated with long-term breast cancer risk in an age-dependent manner. These findings highlight the potential of metabolomics to identify metabolic signatures associated with cancer risk and suggest that age-specific metabolic profiles may be critical for understanding disease etiology and developing targeted prevention strategies[22].

Zhou et al. (2019) compared DNA methylation patterns between incumbent and new-recruit firefighters to identify potential cancer-related epigenetic changes. Blood samples from 45 incumbent and 41 new-recruit firefighters were analyzed using the Illumina Methylation EPIC 850k chip. The study identified four CpG sites with significant differential methylation, with three hypomethylated and one hypermethylated incumbent firefighters. Genome-wide methylation accurately predicted firefighter status and years of service. Pathway analysis linked differential methylation to cancer-related pathways such as Sirtuin signaling, p53 signaling, and AMPK signaling. The study concluded that DNA methylation changes in firefighters are associated with cancer pathways, supporting the hypothesis that occupational exposures contribute to increased cancer risk. This research provides valuable insights into the epigenetic mechanisms underlying firefighter health risks and highlights the potential of DNA methylation as a biomarker for cumulative occupational exposures[23]. Another study by Goodrich et al. (2021) investigates differential DNA methylation patterns between Hispanic and non-Hispanic white firefighters in the United States, focusing on potential epigenetic mechanisms underlying cancer risk disparities. Using the Infinium MethylationEPIC array, the researchers analyzed blood leukocyte DNA from 31 Hispanic and 163 non-Hispanic firefighters. They identified 76 differentially methylated loci, including genes related to xenobiotic metabolism (e.g., SULT1C2) and carcinogenesis (e.g., FOXK2, ZBTB16), with Hispanic firefighters showing lower methylation at several sites, suggesting that epigenetic differences, potentially influenced by genetic ancestry or environmental exposures, may contribute to varying cancer risks among ethnic groups. While the findings are preliminary and limited by sample size, they highlight the need for further research into epigenetic susceptibility and its role in occupational health disparities among firefighters[24].

1.3 Dissertation outline

This dissertation introduces three metabolomics analyses to address the major gaps. Chapter 2 presents our first analysis using urine-based metabolomics on male firefighters responding to structure fires, including an analysis of the effect of Hispanic ethnicity. Chapter 3 introduces another urine-based metabolomics on male firefighters to investigate metabolic responses to WUI fire exposures. Chapter 4 introduces the final urine-based metabolomics analysis with women firefighters responding to training fires. Chapter 5 concludes with a summarization of previous work and future directions.

Chapter 2. Differential Metabolic Profiles by Hispanic Ethnicity Among Male Tucson Firefighters

Abstract

Firefighters face regular exposure to known and probable human carcinogens, such as polycyclic aromatic hydrocarbons (PAHs), benzene, and formaldehyde, possibly contributing to an increased risk of various cancers compared to the general population. Hispanic and black firefighters are at increased risk of additional cancers not elevated in non-Hispanic white firefighters, yet biological pathways underlying these differences are unknown. The study objectives were to evaluate differences in the urinary metabolome between Hispanic and non-Hispanic firefighters, pre-and post-fireground exposure. To investigate the metabolic patterns, we employed a comprehensive metabolomics pipeline that leveraged liquid chromatography coupled with high-resolution mass spectrometry. We applied linear mixed effects regression to identify the differential metabolites at a false discovery rate (FDR) <0.05 among 19 Hispanic and 81 non-Hispanic firefighters. We also performed overrepresentation analysis using Mummichog to identify enriched pathways at FDR <0.05. Out of 175 features in hydrophilic interaction with negative ionization (HILIC(-)) mode and 1847 features in reverse-phase positive (RP(+)) mode, we found 26 and 276 differential urinary features, respectively, when comparing Hispanic and non-Hispanic firefighters. We noted pathway enrichment in tryptophan and galactose metabolism. However, post-exposure, we did not observe differences in the metabolomic response by ethnicity despite differing fireground exposures. Dysregulation in the tryptophan and galactose pathway is an important contributor to cancer risks and may explain the increased cancer risk among Hispanic firefighters.

2.1 Introduction

According to the National Fire Protection Association, there were approximately 1.0 million firefighters in the United States (US) in 2020, of whom 90,000 are women[25]. Firefighters are exposed to known and probable human carcinogens[26], including but not limited to PAHs[13], benzene[13], formaldehyde[27], and phthalates[28], and experience a higher risk for select cancers, including skin melanoma[9], lung[26], leukemia[26], kidney[29], and prostate[9, 29, 30]. In general, firefighters in the US experience excess overall cancer mortality (SMR=1.14; 95% CI 1.10-1.18) as compared to the general US population[1, 10]. Specifically, according to studies of firefighters in Florida[9, 11], Washington[31], California[29, 32], and other US cities[10, 26, 33], male firefighters were at increased risk of skin melanoma, urine tract cancers, mesothelioma and other types of cancers, of which kidney, leukemia, colon, prostate, and testicular cancer have less consistent results in the literature[34]. Consequently, the International Agency for Research on Cancer (IARC) recently classified firefighters' occupational exposure as carcinogenic to humans with sufficient evidence for mesothelioma and bladder cancer[1].

In addition, Hispanic firefighters may be at increased risk for various cancers. In the US, Hispanics experience overall lower all-cancer incidence rate but higher incidence rates of cervical, stomach, liver, and gall bladder cancer than non-Hispanic Whites[35, 36], and Hispanic firefighters may be at similarly increased risk. In a large-scale, case-control study assessing cancer risk among male firefighters, Tsai et al (2015) reported that firefighters of other ethnicity/race (two thirds of whom were Hispanic), as compared to non-Hispanic white firefighters, had statistically significantly increased risks for a total of six cancers, including melanoma, prostate, testicular, bladder, kidney, and brain cancer[8]. We had previously shown that xenobiotic metabolizing genes and cancer-related genes were differentially methylated in Hispanic compared to non-Hispanic firefighters[37]. We also previously showed that firefighters experience differential metabolite expressions at baseline and after fires[38], but did not explore whether these differed by Hispanic ethnicity. Societal conditions within fire departments and potentially differential task assignments to Hispanic firefighters have been hypothesized as possible reasons which might lead to the difference in the cancer risks[8]. Other factors, such as diet and environmental exposure, could also play an important role.

It is unclear, however, whether the biological response to fires varies by ethnicity. In this study, we sought to strengthen our understanding of underlying biological changes explaining cancer risk differences by Hispanic ethnicity using an untargeted metabolomics approach.

Coupled with a baseline and post-fire exposure sampling scheme, we profiled and compared untargeted urinary metabolite profiles in firefighters of Hispanic and non-Hispanic ethnicity. We hypothesized that metabolic profiling would reveal differences in the urine metabolome of Hispanic and non-Hispanic firefighters and that the differences might reveal biological implications for cancer risk disparity by ethnicity. Results from this study may serve as a resource for advancing understanding of cancer risk differences among firefighters by ethnicity and provide a direction for future mechanistic or longitudinal studies.

2.2 Methods

2.2.1 Study population & sample preparation

Our analysis included 100 male firefighters from the Tucson Fire Department. Detailed study recruitment and urine sample collection were reported previously[39]. Briefly, urine samples were gathered from firefighters who had not participated in a fire response for at least four days (baseline). Subsequently, the same firefighters responded to a structural fire (post-fire) and urine samples were collected 2-4 hours afterwards. The urine samples were transported on ice from the Tucson Fire Department to the University of Arizona. The specific gravities (SGs) were measured by refractometry, and samples aliquoted and stored at -80 °C. Urine samples were prepared by spiking a 13C labeled internal standard mix to a 1:1 solution of urine and ice-cold acidified methanol. The product mixtures were vortexed, centrifuged, and extracted in duplicate and stored at -80°C until further analysis. Several quality control samples (QC) and blanks were employed for machine calibration.

2.2.2 High-resolution metabolomics

High resolution metabolomics (HRM) analysis was performed in two sample sets within a three-month period, using liquid-chromatography with high-resolution mass spectrometry (LC-HRMS; Thermo Scientific Exploris Orbitrap 480 Thermo Scientific, Waltham, MA). Duplicate sample extracts were randomized into batches across the two sample sets, except for those from Hispanic firefighters (N=21) which were all analyzed during the first set. To boost metabolic feature coverage, sample extracts were analyzed in two modes using a dual-column, dualpolarity approach that included reverse-phase (RP) C18 chromatography with positive electrospray ionization (ESI) (RP(+)), along with hydrophilic interaction (HILIC) chromatography with negative ESI (HILIC(-)).

We performed HRM following adapted methods from Najdekr et al[40]. Briefly, after injecting 1.0 µL of sample, we conducted RP separation using a 1.8 µm, 2.1 x 150mm HSS T3 Column (ACQUITY Premier HSS T3 Column) and a methanol gradient (A = 99.9% water, 0.1% formic acid; B = 99.9% water, 0.1% formic acid). This involved an initial 3-minute period with 99% A and 0.1% B, followed by a linear increase to 50% B at 11 minutes and a subsequent increase to 95% B held for 2 minutes. For HILIC separation, a 1.7 µm, 2.1 mm x 150 mm Amide column (Waters ACQUITY Premier BEH Amide Column) was employed. The mobile phase consisted of a gradient of 10 mM ammonium formate and acetonitrile (A = 10% water, 90% ACN, 10 mM ammonium formate, 0.1% formic acid; B = 50% water, 50% ACN, 10 mM ammonium formate, 0.1% formic acid). Like RP, the HILIC separation began with a 3-minute period of 99% A and 0.1% B, followed by a linear increase to 50% B at 11 minutes and an additional increase to 95% B held for 2 minutes. The flow rate of the mobile phase was maintained at a constant 0.3 mL/min for both modes. The mass spectrometer operated at a resolving power of 60,000 with a mass-to-charge ratio (m/z) range of 65–1000 Da.

Lab blanks and standard QCs were run before AcquireX samples at the beginning of each batch. A pooled "total" QC sample was run after every 30 sample injections to access instrument variability and aid in normalization. "Batch" QC's were run at the end of the corresponding batch. Finally, a standard library QC was run every 30 samples to access retention time variation during the annotation process. Additionally, the mass spectra of the internal standards were manually inspected using the software Skyline[41] to access batch effects.

2.2.3 Metabolite annotation

Metabolic features were uniquely defined by their mass-to-charge ratio (m/z), retention time and relative abundance. Annotation was accomplished using Compound Discoverer 3.3 (Thermo Scientific). To ensure annotation quality, a mass tolerance of 5 ppm was appliedMass spectra were then annotated against both in-house and online libraries. Annotations were prioritized as follows: 1) in-house standards library (MetaSci, Inc), 2) MzCloud, 3) Masslist, 4) ChemSpider, 5) Metabolika, At the time of this analysis, 300 metabolites from the MetaSci library were confirmed using our in-house library.

Annotation algorithms tend to forcefully search for matches at the risk of generating false positives. We thus limited the mass difference to be less than 5 ppm. To comply with the reporting standards for metabolite annotation[42] and indicate annotation strength, we adopted a modified confidence score for annotation using a self-defined match strength with the in-house database (MzVault), and the online databases (MzCloud, Chemspider, Metabolika, Masslist). The scoring framework was as follows: we assigned a score of 5 for MzVault and MzCloud, 4 for Chemspider, 3 for Metabolika, and 3 for Masslist to the feature if a full match with respective libraries was achieved; we assigned a score of 2 to the features if a partial match was achieved; if no match, we then assigned a score of 0 to the feature. As a result, each feature had 5 scores for relevant libraries, and the sum was interpreted as the overall annotation confidence.

2.2.4 Data preprocessing and statistical analysis

Ion intensity missing values were imputed using random forest algorithm as implemented in the Compound Discoverer Software (Thermo Scientific, Waltham, MA). Biological variation from individual levels of hydration was removed by multiplying the normalized ion intensities by corresponding specific gravity factors (SGF) defined as $SGF = \frac{1.02-1.00}{SG-1.00}$ [43]. Since our samples were analyzed in two sets, we visually checked by mapping out the expression level of lab standards and adjusted for potential batch-wise variation using the NormalizeMets[44] package in R, if any. All metabolic features' ion intensities were then log2 transformed. Median scaling was applied to remove unwanted variation as implemented in the R package NormalizeMets[44].

We calculated the percentage of missing value and a coefficient of variation (CV) by taking the average of duplicate ion intensities for each metabolic feature. We then applied a filtration of missing percentage ≤ 75% and CV ≤ 0.20 to all metabolic features.

An analytical pipeline for our study is outlined in Appendix A Figure A.1. To identify potential differential expression patterns by ethnicity, we drew side-by-side heatmaps with preprocessed expression signals and grouped them by ethnicity category. Metabolic features with annotation confidence > 0 that passed the filtration requirements described above were included in subsequent statistical analyses. Additionally, to identify potential batch effects, we mapped the total abundance by sample injection order/time and visually inspected whether there were clear trends across batches or overtime. We treated preprocessed metabolites' relative abundance as the response and Hispanic ethnicity (yes/no) as the main predictor while adjusting for BMI (kg/m2), age (years), years of firefighting (years), rank, and sample type (baseline and post-fire), and fit a linear mixed effects model with a random effect for participant to account for the repeat sampling from the participants. In the actual differential analysis, we conducted a complete-case analysis and excluded observations with missing demographic values from the model. We defined differential metabolites by a p-value of ≤ 0.05 for the Hispanic term in the mixed model. Multiple testing was adjusted by applying false discovery rate (FDR) adjustment at 0.05 to control for false positives. Metabolites with FDR < 0.05 and annotation confidence score ≥ 10 were presented in table and used to plot the heatmap to discern potential metabolic profile patterns by Hispanic ethnicity.

To evaluate whether biological response to ethnicity varied by fireground exposure status, we evaluated interactions between Hispanic ethnicity and fireground exposure status (baseline, post-fire). We added this interaction term to our main model and used an FDR cutoff of 0.05 to identify significant interactions. Outliers and influential observations were also inspected.

2.2.5 Pathway analysis

We performed pathway analyses for both HILIC(-) and RP(+) samples to investigate the biological implications gleaned from the difference in metabolic profiles by ethnicity, using Mummichog (version 2)[45] as implemented on MetaboAnalyst (version 6.0)[46]. Mummichog uses a set of permutation-based computational algorithms that take advantage of the collective power of metabolic networks to identify overrepresented pathways without metabolite identification from user input, and therefore accelerates the metabolomics workflow[45]. All annotated metabolic signals were included in the pathway analysis as the reference set. P-values from the main model were provided with signals that passed our filtration and a p-value of 1 was provided for those that did not pass. To reduce false positive matches, we restricted all annotated metabolites to be matched in primary ions, and only pathways with minimum size of 3 were investigated. We defined the significance level as a p-value of 0.05. Only significant pathways with p-value < 0.05 were investigated. Overrepresentation analysis results were interpreted in conjunction with pathway maps from Kyoto Encyclopedia of Genes and Genomes (KEGG)[47].

Data preprocessing, statistical analyses, and visualization were performed in the R programming environment, version 4.3.0[48].

2.3 Results

Participant demographics including age, BMI, rank, and years of firefighting experience are listed in Table 1. A total of 100 firefighters from Tucson Fire Department were recruited and 200 urine samples (100 baseline, 100 post-fire) were extracted and measured in duplicate, which gave 399 usable urine extracts (200 baseline; 199 post-fires, 1 sample loss during sample preprocessing). The mean age of participants was 40.05 (9.91) years for Hispanic firefighters (N=19) and 37.07 (8.38) years for non-Hispanic firefighters (N=81). Hispanic firefighters had a non-significantly higher mean BMI score compared to non-Hispanic firefighters in our sample population, 28.53 and 27.68, respectively. Although there were no significant differences by Hispanic ethnicity across rank (captain, engineer, firefighter, trainee, and paramedic) categories, Hispanic firefighters had a larger proportion of engineers and a lower percentage of captains and firefighters than non-Hispanic firefighters. Hispanic firefighters also had more years of experience than non-Hispanic firefighters (10.58 vs 8.76 years), though the difference was not statistically significant.

Table 2.1: Summary statistics of male Tucson Fire Department firefighters.

	Hispanic (N=19)	Not Hispanic (N=81)	P-value
Age	40.05 (9.91)	37.07 (8.38)	0.1811
BMI			0.2791
N-Miss	0	1	
Mean (SD)	28.53 (2.29)	27.68 (3.21)	
Rank			0.6152
Chief/Engineer	9 (47.4%)	33 (40.7%)	
Firefighter	10 (52.6%)	48 (59.3%)	
Years as firefighter			0.3051
N-Miss	0	1	
Mean (SD)	10.58 (6.91)	8.76 (6.90)	

2.3.1 Metabolomics analysis

Combining baseline and post-exposure urine samples, metabolomics analysis detected 175 and 1,847 potential metabolites by HILIC(-) and RP(+) mode, respectively. Metabolites were first identified by comparison to an in-house library containing 300 confirmed metabolites (MzVault). To augment metabolite annotation, we incorporated online libraries, including MzCloud, Chemspider, Metabolika, Masslist. The annotation score scheme was reported previously[38]. After filtration using annotation confidence, CV, and missingness, a total of 137 and 1,007 features from HILIC(-) and RP(+) mode, respectively, were fed into the linear mixed effects model.

2.3.2 Differential analysis

After adjustment for age, BMI, rank, and years in firefighting service, we identified 26 and 276 metabolites from HILIC(-) and RP(+) mode respectively, that were differentially expressed by ethnicity by a p-value ≤ 0.05. Of these, 10 and 109, respectively, remained significant after adjustment for multiple testing. Metabolites that met the FDR < 0.05 and high annotation confidence (≥ 10) cutoffs are presented in Table 2.2. Out of 26 differential metabolites from HILIC(-) mode, 14 were upregulated and 12 downregulated; out of 276 differential metabolites from RP(+) mode, 191 were upregulated and 85 downregulated, both at raw p-value 0.05 level (Figure 2.1). After adjusting for multiple testing by controlling FDR at 5% level, 10 metabolites remained statistically differential from HILIC(-) mode, including d-Sorbitol, sebacic acid, 5-morpholino-2,4(1H,3H)-pyrimidinedione, L-Homocitrulline, Deoxyguanidinoproclavaminic acid, 3-alpha-20-alpha-dihydroxy-5-beta-pregnane3-glucuronide, Diethyl tartrate, val-glu, Perchloric acid, and 6-Hydroxy-3,6,9-trimethyl-2-oxo-2,3,3a,5,6,9b-hexahydro-4H-furo[3',4':6,7]cyclohepta[1,2-b]furan-4-yl acetate; 109 metabolites remained statistically differential from RP(+) mode.

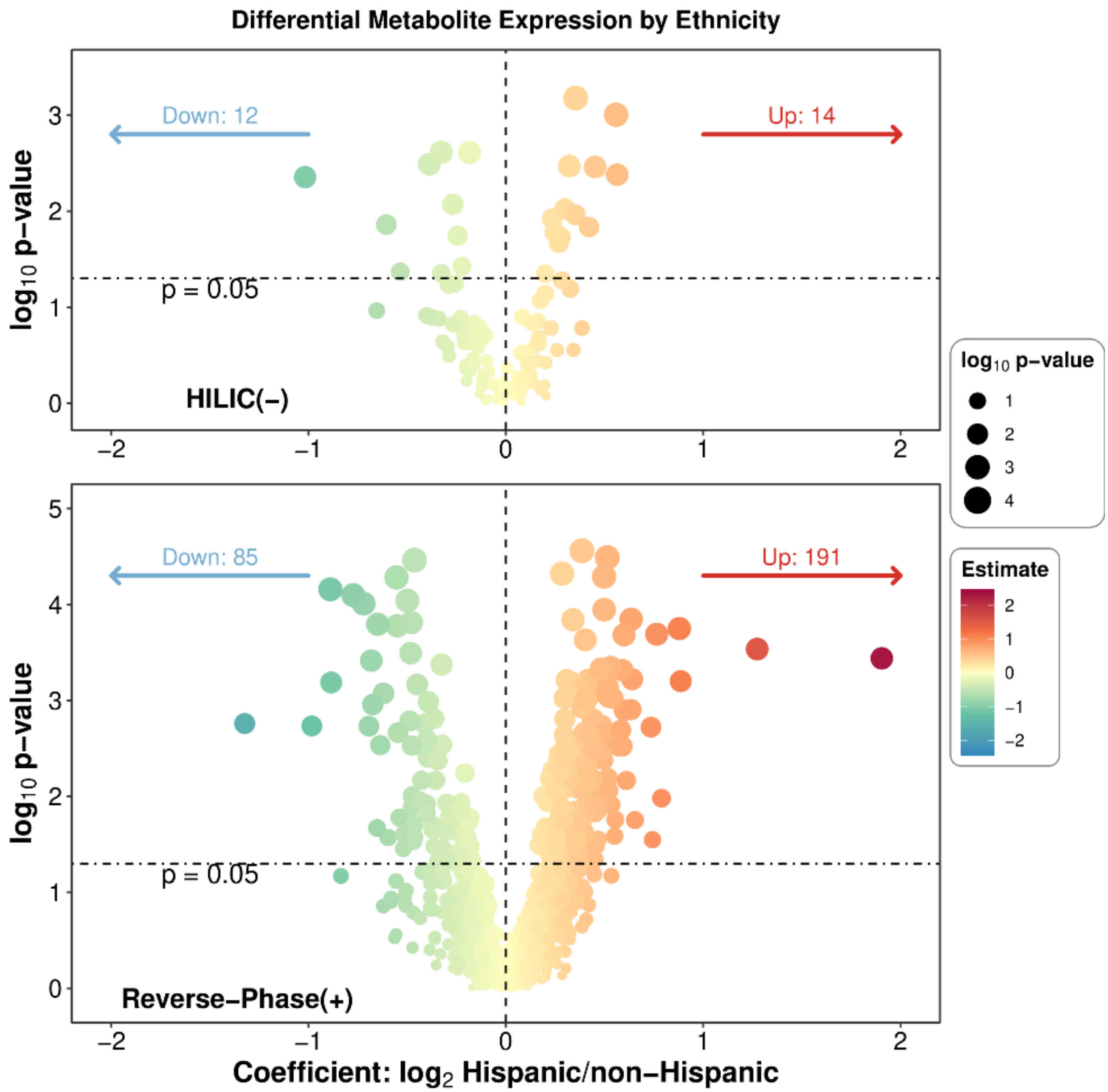


Figure 2.1: Volcano plot for differential metabolites comparing Hispanic to non-Hispanic (reference group) firefighters. Estimate refers to the coefficient estimation for ethnicity term in the linear mixed effects model with random effect for participant, where the coefficient estimates are equal to log2 ratio of the relative abundance of Hispanic to non-Hispanic samples; direction of the coefficients implies whether the metabolite was up- or down-regulated. Models were

controlled for age, BMI, rank, and years of firefighting. P-values were log-10 transformed for visualization purposes, and metabolites with smaller p-values have larger dot sizes.

Table 2.2: Differential metabolites for Hispanic compared to non-Hispanic Firefighters (N=100 participants, 199 samples, 197 complete cases used in the main model).

Mode	Metabolite	Formula	MW	RT [min]	Annotation confidence	Slope	P-value	FDR
HILIC(-)	5-morpholino-2,4(1H,3H)-pyrimidinedione	C8H11N3O3	197.080	10.764	10	0.386	0.000	0.006
HILIC(-)	D-Sorbitol	C6H14O6	182.079	8.672	14	0.559	0.001	0.027
HILIC(-)	Sebacic acid	C10H18O4	202.120	1.520	12	-0.328	0.002	0.047
RP(+)	Cytosine	C4H5N3O	111.043	5.645	13	0.339	0.000	0.007
RP(+)	α-Aspartylphenylalanine	C13H16N2O5	280.106	9.581	12	0.635	0.000	0.007
RP(+)	6-Methoxyquinoline	C10H9NO	159.068	5.991	10	0.403	0.000	0.009
RP(+)	OPEO	C16H26O2	250.193	19.555	10	-0.682	0.000	0.012
RP(+)	α-Aspartylphenylalanine	C13H16N2O5	280.106	9.255	12	0.518	0.001	0.018
RP(+)	N-Acetyl-L-arginine dihydrate	C8H16N4O3	216.122	2.136	12	0.438	0.002	0.026
RP(+)	Oxybenzone	C14H12O3	228.079	14.255	10	-1.324	0.002	0.027
RP(+)	HU-331	C21H28O3	328.204	16.103	10	0.486	0.002	0.027
RP(+)	Methylimidazoleacetic acid	C6H8N2O2	140.059	1.394	12	0.318	0.003	0.035
RP(+)	L-Norleucine	C6H13NO2	131.095	3.815	10	0.487	0.003	0.037
RP(+)	5-Aminolevulinic acid	C5H9NO3	131.058	3.297	15	0.363	0.004	0.038
RP(+)	Cantharidin	C10H12O4	196.074	13.487	12	0.272	0.004	0.039

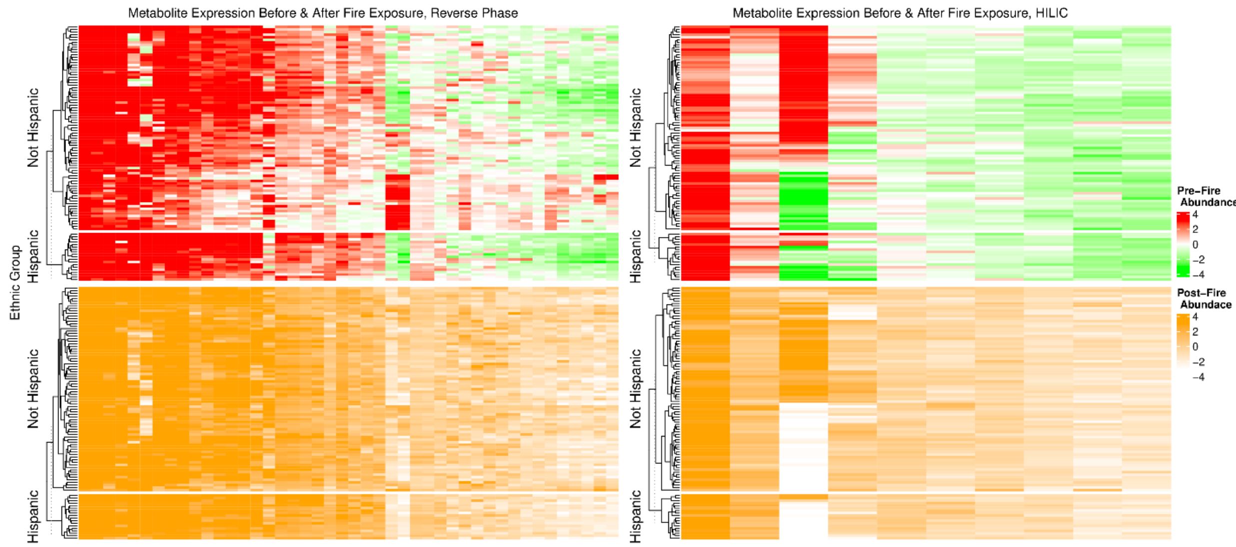


Figure 2.2: Heatmap for differential metabolites with FDR < 0.05 by ethnicity and fire exposure status. The subplot on the left refers to the reverse phase mode and the one on the right refers to the HILIC mode. For both subplots, the expression pattern for differential metabolites were first plotted for pre-fire exposure, on top of post-fire exposure. Pre-fire comparison was drawn in red-green scheme and post-fire comparison was drawn in white-yellow scheme. The relative abundance for each feature is in log2 and centered.

In the heatmaps for the differential metabolites' expression at FDR <0.05 level by ethnicity group (Figure 2.2), we observed clear differences in the metabolite expression profiles comparing Hispanics to non-Hispanics. This difference was consistent regardless of fire exposure status (pre-, and post-fire). A combination of lollipop plot and side-by-side boxplot showing the magnitude, effect size, and direction of the difference in metabolite expression by ethnicity group, (Appendix B Figure B.1, Figure B.2) was attached.

2.3.3 Pathway analysis

The pathway over-representation analysis identified 28 potentially relevant biological functions comparing Hispanic to non-Hispanic firefighters (Figure 2.3). Among these, 2 were associated with HILIC(-), and 26 were linked to RP(+) modes. At 0.05 level, only 2 metabolic pathways remained significantly enriched. Specifically, tryptophan metabolism from RP(+) mode exhibited an enrichment factor of 2.21, while galactose metabolism from HILIC(-) mode showed a substantial enrichment factor of 53.00. The galactose pathway stayed significantly enriched after adjustment for multiple testing.

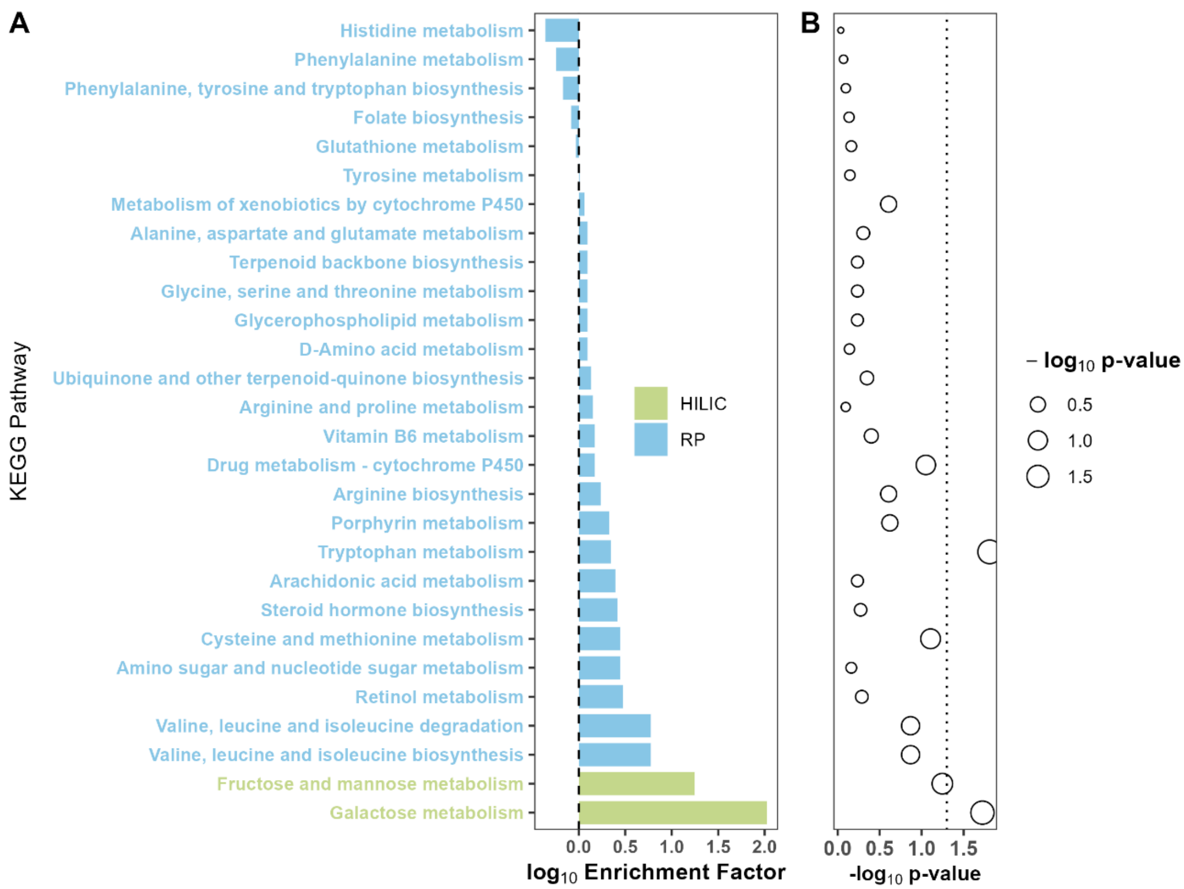


Figure 2.3: Pathway overrepresentation analysis of Hispanic versus non-Hispanic firefighters based on mixed effects model coefficients and statistical significances; A) HILIC(-) and RP(+) mode are colored in green and blue, respectively; dashed line indicates enrichment factor=1, where enrichment factor is calculated as the ratio of the number of significant hits from user input to the expected hits within a pathway, and enrichment factor below 1 indicates the pathway is underrepresented and over 1 overrepresented; B) the p values are calculated by Fisher test

against null hypothesis; the dotted line indicates p-value=0.05 and bigger circle size indicates a low p-value and thus higher significance.

2.3.4 Sensitivity analysis

Sensitivity analyses results indicated no statistically significant interaction between the ethnicity effect and the exposure effect at 0.05 FDR level. Refitting the model after identifying and removing outliers did not substantially change the associations between ethnicity and metabolites' relative abundance. For batch effects, we plotted the relative abundance by samples and observed only random fluctuations in the expression pattern, for both intra and inter batch, by sample injection, and there was no clear pattern in the expression pattern across the two sets.

2.4 Discussion

In this study, we performed untargeted metabolomics to increase the understanding of differential health risk by ethnicity among male firefighters. This approach allowed us to profile the metabolome to identify differential expressions across 100 firefighters. Out of 175 HILIC(-) and 1847 RP(+) matched urinary metabolites, 26 from HILIC(-) and 276 from RP(+) were differentially expressed by Hispanic ethnicity. These differences were enriched for galactose metabolism and tryptophan metabolism pathways. However, the metabolomic response by Hispanic ethnicity did not vary by fireground exposure status, suggesting that differences by ethnicity may not be strongly related to differential responses to fires. Identification of these metabolites and pathways may help explain the increased risk for some cancers by Hispanic ethnicity among firefighters.

In pathway analyses, d-sorbitol and galactitol were significant hits for the galactose metabolism pathway. D-sorbitol is a sugar alcohol obtained by reduction of glucose that has been commercially produced in packaged food and beverages as a sugar substitute. In our analysis, d-sorbitol was found in Hispanic firefighters at a statistically higher level than in non-Hispanic firefighters (slope=0.56, $\frac{Hispanic}{non-Hispanic} = 2^{0.56}$ =1.47), which could be indicative of dietary differences between Hispanic and non-Hispanic firefighters. Galactitol was identified by compound matching of Mummichog. Galactitol is a reduction product formed from excess galactose in a reaction catalyzed by aldose reductase. Galactose is the hydrolysis product of lactose that is found primarily in dairy products. Studies have shown that chronic galactose exposure induces neurodegeneration and oxidative damage as well as acceleration of aging in mice[49, 50]. Different levels of d-sorbitol and galactitol together could indicate differential exposures to environmental factors among firefighters such as dietary quality that chronically might contribute to the cancer risks observed across race/ethnicity, as discussed by Tucker KL et al (2019). Dietary factors have been one of the major contributing risk factors for select cancers including colon, breast, and prostate, in addition, approximately 33% of cancers in the West could be associated with dietary factors[51, 52]. Although evidence of artificial sweeteners and cancer risk is weak and inconsistent[53-55], consumption of sugar substitutes could be linked to obesity which in turn is associated with various types of cancers that may help explain the cancer risk disparity among firefighters.

Tryptophan (TRP) is an essential amino acid obtained from dietary protein that plays an important role in various physiological activities including immunity and neurological functions. TRP is primarily metabolized by the kynurenine pathway (KP) catabolized by indoleamine 2,3-dioxygenase (IDO1, IDO2) whereas the rest takes part in the production of serotonin and tryptamine[56, 57]. IDO was reported to be capable of suppressing T-cell activity by inhibiting T cell proliferation and thus restricting antitumor immune responses in pregnant mice[56, 58]. Disturbance of TRP metabolism, specifically TRP degradation catalyzed by IDO, thus can induce conditions ranging from neurodegeneration to cancer[56, 59, 60]. Our pathway analysis indicated a higher level of TRP and 5-Hydroxy-L-TRP among Hispanic firefighters than non-Hispanic firefighters (Figure 2.4), which could be indicative of difference in intake of TRP from diet. However, the level of TRP is the product of dietary intake and innate TRP metabolism. If the difference in TRP levels persists over time, this could serve as a cue or starting point for cancer risk difference by Hispanic ethnicity among firefighters and a potential target for intervention.

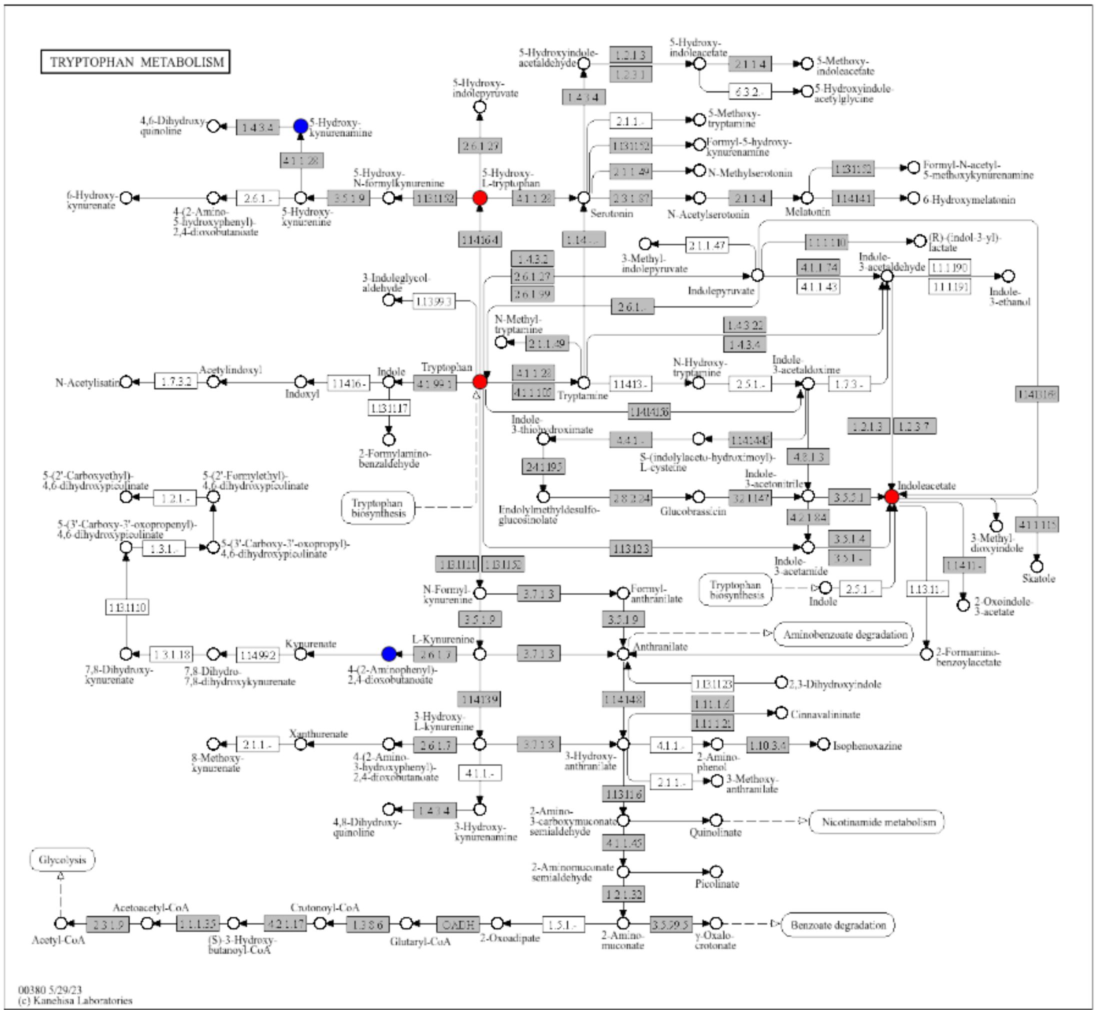


Figure 2.4: Pathway overrepresentation analysis of Hispanic versus non-Hispanic firefighters based on mixed effects model coefficients and statistical significances; A) HILIC(-) and RP(+) mode are colored in green and blue, respectively; dashed line indicates enrichment factor=1, where enrichment factor is calculated as the ratio of the number of significant hits from user input to the expected hits within a pathway, and enrichment factor below 1 indicates the pathway is underrepresented and over 1 overrepresented; B) the p values are calculated by Fisher test

against null hypothesis; the dotted line indicates p-value=0.05 and bigger circle size indicates a low p-value and thus higher significance.

A recent review reported that tryptophan metabolism was among the most consistently perturbed pathways when evaluating association of air pollution and untargeted metabolite expressions, and tryptophan with level-1 identification was reported down-regulated in both serum and plasma untargeted metabolomics studies[61]. In addition, galactose metabolism was reported to be enriched after air pollution in 9 independent metabolomics studies[61]. Although tryptophan was reported in biospecimens other than urine which is different from our bioassay, this could serve as an alternative explanation for ethnic/racial difference in cancer risks among firefighters since firefighters are exposed to carcinogens via inhalation and Hispanic firefighters may experience task assignment with more toxic exposure scenarios than non-Hispanic firefighters.

This work builds upon our previous study that focused on DNA methylation, a relatively stable epigenetic modification that contributes to carcinogenesis when dysregulated. We compared DNA methylation of >700,000 loci between 31 Hispanic and 163 non-Hispanic white firefighters from three US states[24]. We reported DNA methylation differences at 76 loci including in genes involved in xenobiotic metabolism and cancer-related pathways. Altered regulation of these genes in Hispanic firefighters could influence the ability to process and protect against toxic exposures encountered at the workplace or home environment which could in turn influence downstream health risks.

This study also builds on our previous study of metabolic changes following fires in firefighters[38]. In that study, we also observed changes in tryptophan metabolism from baseline to post-fire, along with several other metabolomic changes indicating xenobiotic exposures and impacts on kidney function. In this study, we show that the response to fires does not differ by ethnicity at FDR<0.05, although we may be underpowered to detect consistent interaction effects. However, tryptophan changes may be particularly important for Hispanic firefighters, given the double implication of changes by ethnicity and after fire.

Collectively, enrichment of galactose metabolism and tryptophan metabolism among Hispanic compared to non-Hispanic firefighters may be indicative of dietary differences between the groups or environmental air pollution difference in the workplace or the home. These findings may carry practical implications in terms of cancer risk investigation among firefighters by race/ethnicity, such that difference in diet and exposure to air pollutants may play a critical role in the elevated risks among Hispanic firefighters. Importantly, intervention and prevention strategies could be developed to promote health and potentially reduce differences in external exposures that impact galactose and tryptophan metabolism.

To conclude, male firefighters showed a broad metabolic difference by ethnicity, including altered galactose and tryptophan metabolism that may be indicative of chronic dietary or environmental exposure differences among Hispanic and non-Hispanic firefighters. This interplay could collectively contribute to the differences in cancer risks among male firefighters by Hispanic ethnicity.

Chapter 3. Evaluating Urine Metabolic Profiles with Wildland-Urban-Interface (WUI) Fire Exposure: A Comparison with Municipal Structure Fires (MSF)

Abstract

Firefighters have frequent exposure to carcinogens and an increased risk of cancer. Wildland-urban interface (WUI) fires, which involve both structures and undeveloped wildland fuels, pose unique challenges to the health of firefighters. However, the extent of health risks associated with these fires remains underexplored. This study aims to identify altered urine metabolites and metabolic processes among male firefighters that were associated with WUI fires as compared with municipal structure fires (MSF). Untargeted metabolomic profiling was applied to pre-exposure (baseline) and postfire urine samples collected from firefighters responding to WUI and MSF exposure. Differential analysis was conducted by fitting linear mixed effects regression models on preprocessed ion intensity and exposure status while adjusting for demographic covariates. Differential metabolites by post-exposure status were identified using a false discovery rate (FDR) threshold of <0.05. Enrichment analysis was performed to identify pathways that were significantly perturbed at a Bonferroni adjusted p-value <0.05 level. Eighty-five firefighters contributed paired baseline and post-fire samples from WUI events, and 98 firefighters contributed paired baseline and post-fire samples from MSF events. We performed metabolic profiling on baseline and postfire urine samples from WUI and structure fires using four modes: HILIC(-), HILIC(+), C18(-), and C18(+) and identified metabolites against an in-house library. We identified 244, 297, 320, and 266 level 1 metabolites from the four respective modes. In the statistical analysis, the main model identified a total of 176 differential metabolites from WUI fires. For MSF, the model identified a total of 652 differential metabolites from the four respective modes. Most metabolites with significant changes after a WUI fire also changed significantly after an MSF event. Two pathways were significantly enriched after WUI fires, while seven pathways were significantly enriched after

MSF exposure and two pathways overlapped between the two types of fires. Fire exposure induces numerous metabolic perturbations in firefighters that may partially explain their elevated cancer risks. Although individual metabolites changed in a similar fashion across both WUI and MSF, structure fires were associated with an increased number of metabolite changes and some of the altered pathways differed between exposures to WUI fires vs. MSF. These results suggest that exposures to WUI fires and MSF present both common and unique cancer risks for firefighters.

3.1 Introduction

The wildland-urban-interface (WUI) is a transitional zone where developed residential and commercial areas intermingle with undeveloped wildland and vegetation. According to the United States Fire Administration[62], urban areas have expanded into wildlands in the continental United States at an annual rate of 809,371 hectares over the past decade, affecting more than 60,000 communities and associated with destruction of an average of 3,000 structures annually by WUI fires. Firefighters are routinely exposed to known and probable carcinogens, such as benzene[13], polycyclic aromatic hydrocarbons (PAHs)[13, 39], and per- and polyfluoroalkyl substances (PFAS) among others during fire responses. These occupational exposures were recently classified as a Group 1 carcinogen by the International Agency for Research on Cancer[1]. Wildland fires burn biomass and produce toxic smoke that induces oxidative stress[63], impairs lung function[64], and causes inflammatory responses[18, 65, 66]. These effects are associated with long-term health manifestations such as cardiovascular diseases[67] and cancers[1, 68, 69]. In addition to airborne contaminants from wildland fires, WUI fires can produce a range of differing yet more toxic chemicals from the combustion of structures and vehicles including hydrogen cyanide (HCN), volatile organic compounds (VOCs), and toxic metals[16, 70, 71]. Furthermore, the structures that are burned in WUI fires produce similar chemicals to wildland fires but at different levels; for example, PAHs from burning structures are emitted at several orders of magnitude greater levels than occurs in wildfires[72].

Due to the mingling of urban environments and natural vegetation in undeveloped wildlands, and the associated complexity of occupational health concerns, WUI fires present challenges for local fire management agencies. Unlike the heavier, insulating personal protective equipment (PPE) used in structure firefighting, which includes a three-layered ensemble of protective textiles and self-contained breathing apparatuses against intense heat and toxic smoke, WUI/Wildland single layering is lighter and less insulating to accommodate a greater need for mobility and breathability in harsh outdoor conditions. Consequently, firefighters responding to WUI events may be exposed to higher doses of toxic chemicals due to the reduced level of protection.

While previous research has characterized chemical exposures for firefighters[13, 16, 28, 39, 63, 70-72] and presented epidemiological evidence of health effects associated with occupational firefighting[9, 26, 33, 67, 73], exposures from WUI fires and their associated health effects have been understudied. It remains unclear how WUI fires affect the metabolic equilibrium of firefighters and how this could contribute to chronic health conditions. In particular, understanding how the metabolomic response to WUI fires differs from the response to structure fires can help identify biological mechanisms that may contribute to differing risks.

Using untargeted, high-resolution metabolomics (HRM), this study aims to systematically evaluate the impact of WUI fires on the metabolic profiles of firefighters and to explore associated health risks, as well as to compare the biological effects of exposure to WUI fires with exposure to municipal structure fires (MSF) alone. We hypothesize that a set of altered metabolites exist that are associated with adverse health outcomes for firefighters. Furthermore, we anticipate that WUI fires and MSF share common metabolic disruptions, while each induces distinct alterations in metabolic functions that pose unique health risks specific to each fire type.

3.2 Methods

3.2.1 Study population and sample collection

The study population included 87 firefighters enrolled in the Fire Fighter Cancer Cohort Study (FFCCS) from two Southern California county fire departments whose response duties include both MSF and WUI fires, using a baseline and post-exposure sampling design. This study also included 100 men firefighters responding to MSF from the Tucson Fire Department to provide a comparison with firefighters responding to structure fires alone. For WUI firefighters, all baseline urine samples were collected in September 2019 except for two samples collected in May 2019, and all post-fire samples were collected within 3-5 hours of ending a work cycle during a WUI fire deployment in October 2019. All MSF urine samples were collected between October 2015 and December 2018. Baseline and postfire urine samples from structure firefighters were analyzed in the same laboratory environment as were the WUI samples. Urine samples were transported on ice and stored at -80°C at the University of Arizona until processing and analyses.

3.2.2 Sample preparation

Urine samples were processed in batches that included both study samples and quality assurance/quality control (QA/QC) samples using an Opentron OT2 automated liquid handler and 96-well plates. Before analysis, the samples were thawed at 4ºC. A 30 µL aliquot of urine was then mixed with 90 µL of acetonitrile containing 13C-labeled internal standards. The mixture was vortexed for 2 minutes, left to equilibrate at 4ºC for 30 minutes, and subsequently centrifuged at 3,220×g for 45 minutes at 4ºC. Following centrifugation, two 30 µL aliquots of the supernatant were transferred to 96-well plates, each containing either 60 µL of water (for Reverse Phase Chromatography (C18)) or 60 µL of a 1:1 acetonitrile/water solution (for Hydrophilic Interaction Liquid Chromatography (HILIC)). These were vortexed for another 2 minutes and stored in a refrigerated autosampler until they were analyzed.

3.2.3 High-resolution metabolomics

All WUI urine samples were sent on ice to the Comprehensive Laboratory for Untargeted Exposome Science at Emory University for high-resolution metabolic profiling. Our previous study on MSF was profiled using a different HRM platform. To facilitate the WUI and MSF comparison, we re-analyzed urine samples collected from firefighters before and after MSF exposure using the same HRM platform as WUI fires and compared these data with new analyses of urine samples collected from firefighters before and after their deployment against WUI fires.

HRM was performed using two systems configured for either C18 or HILIC. These systems consisted of a Vanquish Duo Ultra Performance Liquid Chromatography (UPLC) unit (Thermo Fisher Scientific, Rockford, IL, USA) paired with an Exploris 120 High-Resolution Mass Spectrometry (HRMS) system (Thermo Fisher Scientific, Rockford, IL, USA). The LC column temperatures were maintained at 40°C for HILIC and 30°C for C18, while the autosampler was kept at 5°C. Samples underwent analysis via dual column chromatography with mobile phases tailored for optimal positive or negative ionization. For C18 chromatography, a Higgins TARGA C18 5µm 50x2.1mm column (Higgins Analytical, Inc., Mountain View, CA, USA) was used in both positive and negative ionization modes. HILIC chromatography utilized a SeQuant ZIC-HILIC 3.5µm 50x4.6mm column (Merck KGaA, Darmstadt, Germany) for positive mode and an XBridge Amide 3.5µm 3.0x50mm column (Waters Corporation, Milford, MA, USA) for negative mode. The mobile phase (MP) for C18 analysis consisted of water with 0.1% formic acid (MP-A) and acetonitrile with 0.1% formic acid (MP-B) for positive mode, and 10mM ammonium acetate in water (MP-B) paired with a 97.5/2.5 (v/v) acetonitrile/water mixture (MP-A) for negative mode. For HILIC, the mobile phases included water with 0.1% formic acid (MP-B) and acetonitrile with 0.1% formic acid (MP-A) for positive mode, and 10mM ammonium acetate in water at pH 9.5 (MP-B) with a 97.5/2.5 (v/v) acetonitrile/water mixture (MP-A) for negative mode. Flow rates ranged from 0.3 mL/min to 0.6 mL/min, with a total run time of 7.5 minutes.

Resolutions for MS and ddMS2 were set at 120,000 FWHM (at m/z 200, at 3 Hz) and 30,000 FWHM (at m/z 200, at 12 Hz), respectively; internal calibration was performed scan-to-scan. The scan range was set from 85-850 m/z. The automatic gain control (AGC) target and maximum injection time in full-scan MS settings were set to Standard and Auto, respectively. This allowed the instrument to automatically adjust the injection time for an ion count of ~1 × 106 for MS and 1e5 for ddMS2. The TopN (N, the number of topmost abundant ions for fragmentation) was set to 4, and collision energy (NCE) was set to 20, 40, and 60. A heated electrospray ion (ESI) source was used. The spray voltage was set at 3.5 kV for positive mode and 2.5kV for negative mode. The capillary temperature and the auxiliary gas heater temperature were set at 325 and 350 °C, respectively. Sheath gas and auxiliary gas flow rate were set at 50 and 10 (in arbitrary units), respectively. The Funnel RF level was set to 70.

3.2.4 Feature preprocessing and metabolite annotation

Following analysis of all study and QA/QC samples, raw instrument files were converted to mzXML[74] and extracted using the two-stage hybrid feature detection and alignment procedure available in apLCMS[75] using five parameter settings optimized for a range of peak intensities. The resulting feature tables were merged using xMSanalyzer[76] and batch-corrected using ComBat[77]. Metabolites were identified by comparing detected m/z and retention time to an in-house database of 1,200 standards analyzed using the same method parameters that included a wide range of environmental and endogenous compounds. Metabolite identifications were determined by matching mass-to-charge ratio (m/z) and retention time with a tolerance of 5ppm and 15 seconds, respectively. Metabolic features were uniquely defined by their m/z, retention time, and ion intensity.

3.2.5 Data processing and statistical analysis

Before data processing and statistical analysis, the overall composition of identified metabolites was plotted. We restricted all analyses to metabolites annotated with level 1 confidence[42, 78] and present in at least 75% of the samples. These metabolites then underwent missing value imputation and further analyses. Missing values in metabolomics studies can arise due to values being below the detection limit and/or the absence of the metabolite[79]. Missing values on ion intensity were imputed using a random forest algorithm as implemented in the R package missForest[80, 81]. All ion intensities of metabolites were log10-transformed and standardized to stabilize variation and meet linear model assumptions.

Considering potential disadvantages of uneven cluster sizes[82], and to facilitate direct comparison of results between firefighters responding to WUI and MSF (where a 1:1 matched sampling scheme by participant was implemented[38]), we included 1:1 matched baseline-postfire urine sample pairs from firefighters responding to WUI fires for regression analysis. Regression analysis was performed using complete cases. For WUI samples, a linear mixed effects model with random intercept for each firefighter was fitted for each urinary feature with preprocessed ion intensity being the response and fire exposure status (baseline vs. post-fire) being the main predictor, while adjusting for covariates including age, years of experience, Hispanic ethnicity, and rank at study enrollment. The same model was fitted on MSF samples. We adjusted for multiple testing by controlling the FDR at 0.05 level, and we defined differential metabolites as those with FDR $q < 0.05$. The differential status was presented and compared both graphically and in tabular format. All preprocessing and statistical analyses were performed in the R programming environment (version 4.3.2)[48].

3.2.6 Pathway analysis

We performed enrichment analysis for each chromatography-ESI mode to investigate changes in metabolic profiles associated with WUI and MSF exposure at the metabolic pathway level. Enrichment analysis was conducted using annotated metabolites as implemented on MetaboAnalyst (version 6.0)[83]. Specifically, all annotated metabolites were included as the reference set, and all differential metabolites at the FDR 0.05 level were included as the metabolites of interest. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways[47] were utilized as the pathway sets. To reduce false positive metabolite matches, we restricted interpretation to pathways significantly enriched with at least three metabolites. To minimize false positives in enriched pathways, we applied Holm-Bonferroni adjustment for multiple testing. We defined enriched pathways as those with an adjusted p-value less than 0.05. Pathway enrichment status was presented and compared in a dot plot facetted by study/fire type. All pathways with raw p-values less than 0.05 were included in the graphical comparison.

3.3 Results

3.3.1 Study population and sample

A total of 87 WUI firefighters contributed 204 urine samples. This group included only one female firefighter, and it also included one male firefighter with only one sample available. We excluded these 2 participants and their corresponding urine samples from statistical analyses, resulting in a final group of 85 men firefighters who contributed 85 urine samples from baseline visits and 117 urine samples from post-fire visits. Given that post-fire samples included repeated samples from some participants following different fire incidents, each baseline sample was paired with the closest post-fire sample in time by participant, resulting in 85 pairs of baseline-postfire samples for WUI firefighters. In the MSF study, 100 men firefighters donated a total of 400 urine samples. Among them, one firefighter had only one visit available, and one had missing demographic information. We excluded samples from these two participants from analyses and paired each baseline sample with the closest post-fire sample in time by participant, resulting in 98 MSF firefighters contributing 98 pairs of baseline-postfire samples.

Firefighters responding to WUI fires had an average age (standard deviation) of 37.1 (10.0) years and an average of 12.2 (9.7) years in firefighting service. Thirty-four percent of the firefighters responding to WUI fires were Hispanic, and more than one-third were in the firefighter/paramedic/emergency medical technician (EMT) category. Nearly 30% of firefighters responding to WUI fires held college degrees, and more than 78% had a history of smoking (defined as having ever smoked at least 100 cigarettes). In comparison, firefighters responding to MSF had an average age of 37.5 (8.6) years and an average of 9.0 (6.9) years in firefighting service. Nineteen percent of MSF firefighters were Hispanic, 60% were in the firefighter/paramedic/emergency medical technician (EMT) category, and more than 78% had a history of smoking. Overall, firefighters responding to WUI fires and MSF were comparable in age, education, and smoking history. However, firefighters responding to WUI fires had more firefighting experience compared to firefighters responding to MSF.

Table 3.1: Summary statistics of demographics for men firefighters responding to WUI fires (N=85) for the wildland-urban-interface firefighter study and men firefighters responding to MSF (N=98) for the structure fire study.

	WUI (N=85)	MSF (N=98)
Age	37.1 (10.0)	37.5 (8.6)
Time in Firefighting Service (years)	12.2 (9.7)	9.0 (6.9)
Hispanic Ethnicity		
No	56 (65.9%)	79 (80.6%)
Yes	29 (34.1%)	19 (19.4%)
Rank at Sampling		
Captain/Chief/Engineer	21 (24.7%)	40 (40.8%)
Firefighter/Paramedic/EMT[1]	37 (43.5%)	58 (59.2%)
Driver Operator	15 (17.6%)	
Other	12 (14.1%)	
Education		
No. Missing value	0	12
College graduate and higher	26 (30.6%)	30 (34.9%)
Some Colleges or lower	59 (69.4%)	56 (65.1%)
Ever Smoked 100 Cigarettes		
No. Missing value	30	14
Yes	43 (78.2%)	66 (78.6%)
No	12 (21.8%)	18 (21.4%)

3.3.2 High-resolution metabolomics

Using untargeted, high-resolution metabolomics combined with an in-house library of approximately 1,200 environmental pollutants, we detected 244, 297, 320, and 266 urinary metabolites with level-1 annotation confidence in HILIC(-), HILIC(+), C18(-), and C18(+) modes, respectively. These metabolites predominantly consisted of endogenous compounds, including carnitine, bile acids, fatty acids, sterols, and steroid hormones, as well as biomarkers of exogenous chemicals such as pesticides, PFAS, phenols, phthalates, and smoking-related compounds. After excluding metabolites present in less than 75% of urine samples, we retained a total of 179, 255, 262, and 213 differential metabolites from WUI samples, respectively. Similarly, we retained 173, 257, 268, and 215 metabolites from MSF samples. Out of the 218

[1] EMT, emergency medical technician

excluded metabolites, 28 showed significant changes after WUI fire exposure based on a raw p-value of 0.05. However, only 10-methyloctadecanoic acid remained statistically significant at an FDR level of 0.05, likely due to skewness in the post-fire distribution.

3.3.3 Differential analysis

After adjustment for age, Hispanic ethnicity, time in fire service, and rank, the main model identified 16, 51, 66, and 43 differential metabolites for HILIC(-), HILIC(+), C18(-), and C18(+) modes, respectively, when comparing baseline and postfire urine samples of firefighters responding to WUI fires (Figure 3.1). Subsequently, after adjusting for the same set of covariates, our main model identified 104, 191, 208, and 149 differential metabolites for HILIC(-), HILIC(+), C18(-), and C18(+) modes, respectively, from urine samples of firefighters responding to MSF (Figure 3.1).

Table 3.2: Top 10 differential metabolites by slope within each mode comparing baseline to post-fire urine samples with FDR < 0.05 for firefighters responding to WUI fire exposure in comparison with MSF MZ: mass to charge ratio, RT: retention time in seconds, slope: the coefficient estimates for the exposure status (baseline-postfire: 0-1) term of the main model. Positive slopes indicated an increase in ion intensity of metabolites after fire exposure whereas negative slopes indicated a decrease.

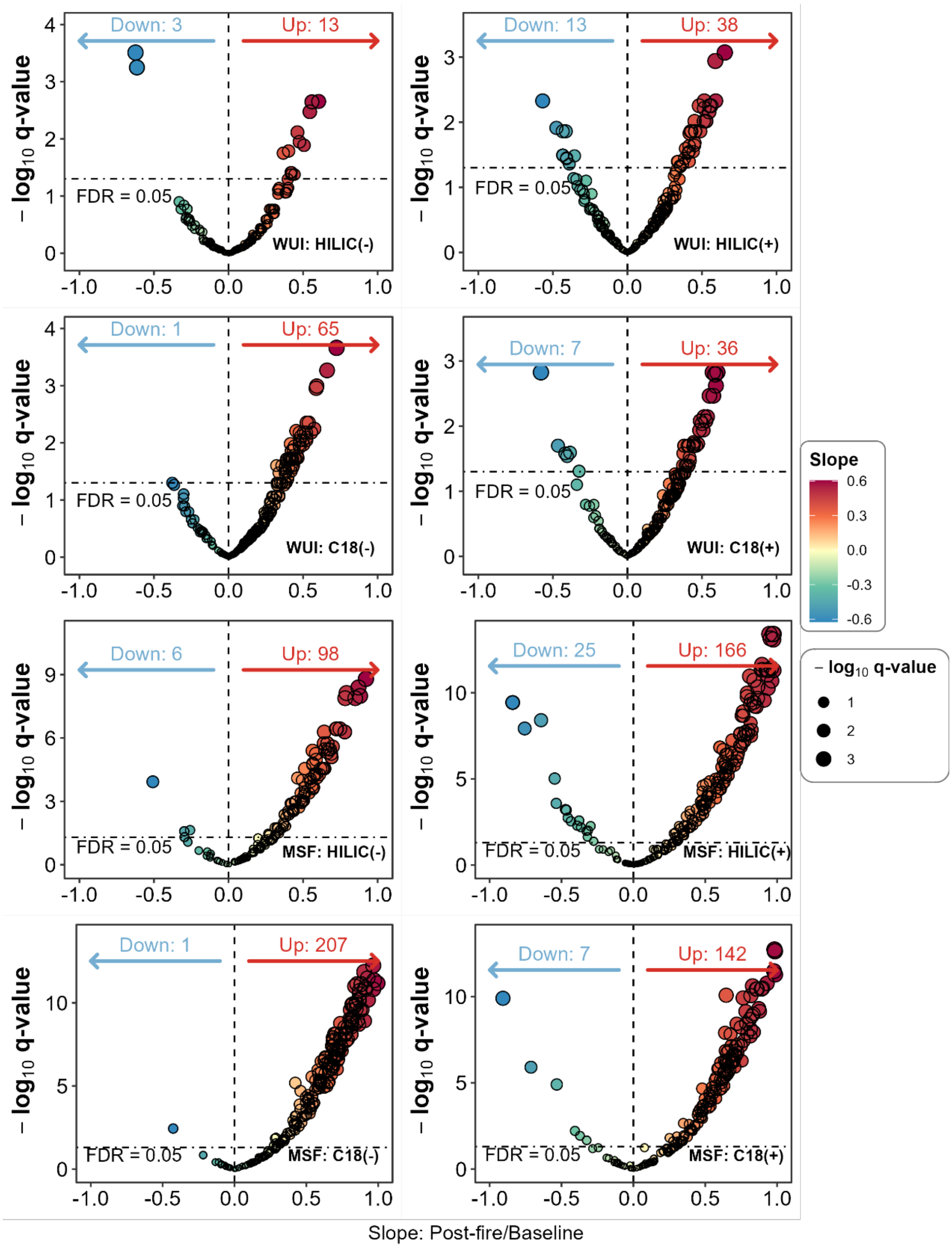
						WUI			MSF		
Mode	**CID**	**Metabolite**	**Formula**	**MZ**	**RT**	**Slo**	**P-**	**FD**	**Slo**	**P-**	**FD**
C18(+	70679	Octenoyl-L-carnitine	C25H47N	286.2	206.8	0.6	<0.0	0.00	0.6	<0.0	<0.0
C18(+	92136	2-Aminoadipic acid	C6H11NO	162.0	24.02	0.5	<0.0	0.00	0.5	<0.0	<0.0
C18(+	15173	5-Hydroxypipecolic acid	C6H11NO	146.0	22.91	0.5	<0.0	0.00	0.4	<0.0	<0.0
C18(+	18189	4-Acetamidobutyric acid	C6H11NO	146.0	29.82	0.5	<0.0	0.00	0.4	<0.0	<0.0
C18(+	774	Histamine	C5H9N3	112.0	17.45	0.5	<0.0	0.00	0.5	<0.0	<0.0
C18(+	90659	Hydroxybutyrylcarnitine	C11H21N	248.1	19.78	0.5	<0.0	0.00	---[2]	---	---
C18(+	27696	1H,1H-Perfluoropentylamine	C5H4F9N	250.0	80.40	0.5	<0.0	0.00	0.4	0.003	0.00

[2] Blank cell

C18(+	43917	Methylthioadenosine	C11H15N	298.0	31.25	0.5	<0.0	0.00	0.8	<0.0	<0.0
C18(+	10917	L-Carnitine	C7H15NO	162.1	22.18	0.5	<0.0	0.00	---	---	---
C18(+	27244	L-Cartinine	C7H15NO	162.1	18.70	0.5	<0.0	0.00	---	---	---
C18(-)	53127	[C10.1]-10-hydroxy-2-decenoic	C10H18O	185.1	55.10	0.7	<0.0	<0.0	---	---	---
C18(-)	31401	Ursodeoxycholate	C24H40O	391.2	229.4	0.6	<0.0	0.00	0.5	<0.0	<0.0
C18(-)	52827	[C8.1]-2-Octenoic acid	C8H14O2	141.0	161.4	0.5	<0.0	0.00	0.6	<0.0	<0.0
C18(-)	92136	Aminoadipate	C6H11NO	160.0	18.90	0.5	<0.0	0.00	0.8	<0.0	<0.0
C18(-)	11039	Monoisononyl phthalate	C17H24O	291.1	230.7	0.5	<0.0	0.00	---	---	---
C18(-)	1028	Prephenic acid	C10H10O	225.0	18.30	0.5	<0.0	0.00	0.5	<0.0	0.00
C18(-)	464	Hippurate	C9H9NO3	178.0	28.00	0.5	<0.0	0.00	0.7	<0.0	<0.0
C18(-)	5960	Aspartate	C4H7NO4	132.0	18.90	0.5	<0.0	0.00	0.7	<0.0	<0.0
C18(-)	18189	4-Acetamidobutanoate	C6H11NO	144.0	19.40	0.5	<0.0	0.00	---	---	---
C18(-)	10972	N-Acetylglycine	C4H7NO3	116.0	19.70	0.5	<0.0	0.00	0.7	<0.0	<0.0
HILIC	16247	Aminocarb	C11H16N	209.1	208.2	0.6	<0.0	0.00	0.6	<0.0	<0.0
HILIC	52809	Gamma-Linolete	C18H30O	279.2	66.00	0.5	<0.0	0.00	0.5	<0.0	0.00
HILIC	15092	3-Methyl-2-Oxindole	C9H9NO	148.0	70.30	0.5	<0.0	0.00	0.5	<0.0	<0.0
HILIC	10917	L-Carnitine	C7H15NO	162.1	294.3	0.5	<0.0	0.00	---	---	---
HILIC	68570	Estradiol-17Alpha	C18H24O	137.0	68.20	0.5	<0.0	0.00	0.9	<0.0	<0.0
HILIC	73671	Urocate	C6H6N2O	139.0	267.8	0.5	<0.0	0.00	0.6	<0.0	<0.0
HILIC	64268	Isovaleryl-L-carnitine	C12H23N	246.1	230.2	0.5	<0.0	0.01	0.3	0.003	0.00
HILIC	53481	Valeryl-L-carnitine	C12H23N	246.1	230.2	0.5	<0.0	0.01	0.3	0.003	0.00
HILIC	64269	2-Methylbutyroylcarnitine	C12H23N	246.1	232.7	0.5	<0.0	0.01	0.3	0.003	0.00
HILIC	43922	Pipecolate	C6H11NO	130.0	265.8	0.5	<0.0	0.00	---	---	---
HILIC	8094	Heptanoate	C7H14O2	129.0	66.70	0.6	<0.0	0.00	0.5	<0.0	<0.0
HILIC	26612	[C10.0]-10-Hydroxydecanoic acid	C10H20O	187.1	74.10	0.5	<0.0	0.00	0.4	0.001	0.00
HILIC	74300	10-Hydroxydecanoate	C10H20O	187.1	83.40	0.5	<0.0	0.00	0.4	0.001	0.00
HILIC	8892	Hexanoate	C6H12O2	115.0	78.31	0.5	<0.0	0.00	0.4	0.001	0.00
HILIC	61743	[C10.1]-9-Decenoic acid	C10H18O	169.1	59.30	0.5	<0.0	0.01	0.5	<0.0	0.00
HILIC	95433	[2-OH,2-Me-4.0]-2-Hydroxy-2-	C5H10O3	117.0	144.0	0.4	<0.0	0.01	0.5	<0.0	<0.0
HILIC	52827	[C8.1]-2-Octenoic acid	C8H14O2	141.0	68.00	0.4	<0.0	0.00	0.5	<0.0	<0.0

HILIC	18189	4-Acetamidobutanoate	C6H11NO	144.0	273.0	0.4	0.004	0.04	0.3	0.009	0.01
HILIC	26613	[C8.0]-3-Hydroxyoctanoic acid	C8H16O3	159.1	72.10	0.4	0.003	0.03	---	---	---
HILIC	971	Oxalate	C2H2O4	88.98	343.2	0.4	0.004	0.04	0.4	0.002	0.00

Overall, we observed a greater number of upregulated than downregulated metabolites in both WUI and MSF samples (Figure 3.1). Specifically for firefighters responding to WUI fires, all 16 differential metabolites identified in HILIC(-) mode were endogenous compounds. Out of the 51 differential metabolites identified in HILIC(+) mode, five were markers of pesticides and the remainder were endogenous compounds. In C18(-) mode, only one phthalate was differentially expressed, with the rest being endogenous metabolites. In C18(+) mode, two pesticides, two PFAS species, and one tobacco use biomarker were differentially expressed. For firefighters responding to MSF, HILIC(-) identified five differentially expressed metabolites associated with pesticides; HILIC(+) identified 17 pesticide metabolites; C18(-) identified one marker for a PFAS, one for a pesticide, and one for phenol; C18(+) identified two markers for PFAS and five for pesticides, with the rest coming from endogenous classes. The top ten differential metabolites for WUI fire exposure by slope for each mode are shown in Table 3.2 along with the same metabolites from MSF exposure. The comparison between WUI and MSF exposure revealed a total of 148 differentially expressed metabolites common to both studies and 27 unique metabolites specific to WUI fires. These unique urinary metabolites consisted mostly of endogenous molecules, along with one marker for a phthalate (monoisnonyl phthalate) and one tobacco use biomarker (nicotine).



Down: 3
Up: 13
FDR = 0.05
WUI: HILIC(-)
Down: 13
Up: 38
WUI: HILIC(+)
Down: 1
Up: 65
WUI: C18(-)
Down: 7
Up: 36
WUI: C18(+)
Down: 6
Up: 98
MSF: HILIC(-)
Down: 25
Up: 166
MSF: HILIC(+)
Down: 1
Up: 207
MSF: C18(-)
Down: 7
Up: 142
MSF: C18(+)
$-\log_{10}$ q-value
Slope
0.6
0.3
0.0
-0.3
-0.6
$-\log_{10}$ q-value
1
2
3
Slope: Post-fire/Baseline

Figure 3.1: Volcano plot of differential metabolites comparing baseline urine samples to post-fire urine samples from men firefighters responding to WUI fires. The slope term was from the main model where exposure status (postfire/baseline) was the main predictor and preprocessed ion intensity was the response. Upregulation is marked in red whereas downregulation in light blue. The horizontal dashed line marked "FDR=0.05" is the boundary that served as the threshold for differential status in this study. FDR q-values were -log10 transformed.

3.3.4 Pathway analysis

Upon restricting the size of pathways to contain a minimum of three metabolites and a raw p-value of 0.05 or lower, the pathway analysis yielded a total of 18 pathways for WUI fires, distributed as follows: one pathway in HILIC(-) mode, two pathways in HILIC(+) mode, 11 pathways in C18(-) mode, and four pathways in C18(+) mode. For structure fires, the pathway analysis identified 49 altered pathways including 12 pathways in HILIC(-) and HILIC(+) mode each, 16 in C18(-) mode, and nine in C18(+) mode. At an adjusted (Bonferroni's method) p-value 0.05 level, two metabolic pathways were reported in samples from firefighters responding to WUI fires including valine, leucine and isoleucine biosynthesis and tyrosine metabolism. In contrast, nine pathways were significantly enriched from MSF samples including biosynthesis of valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, pantothenate and CoA, and arginine, and metabolism of tyrosine, alanine, aspartate, glutamate and purine (Figure 3.2). Perturbations in the biosynthesis of phenylalanine, tyrosine, tryptophan, and pantothenate and CoA were also reported in WUI fire samples, though these did not reach statistical significance. Metabolism and biosynthesis of tyrosine, tryptophan, and phenylalanine were significantly disturbed only in post-fire samples from firefighters responding to structure fires. Valine, leucine and isoleucine biosynthesis and tyrosine metabolism were significantly enriched from both studies at the adjusted $p < 0.05$ level. MSF exposure introduced more significantly disturbed pathways. Notably, the metabolism of purine, alanine, aspartate, and glutamate, and biosynthesis of phenylalanine, tyrosine, tryptophan, and pantothenate and CoA, were perturbed only in samples from MSF firefighters at the adjusted $p < 0.05$ level.

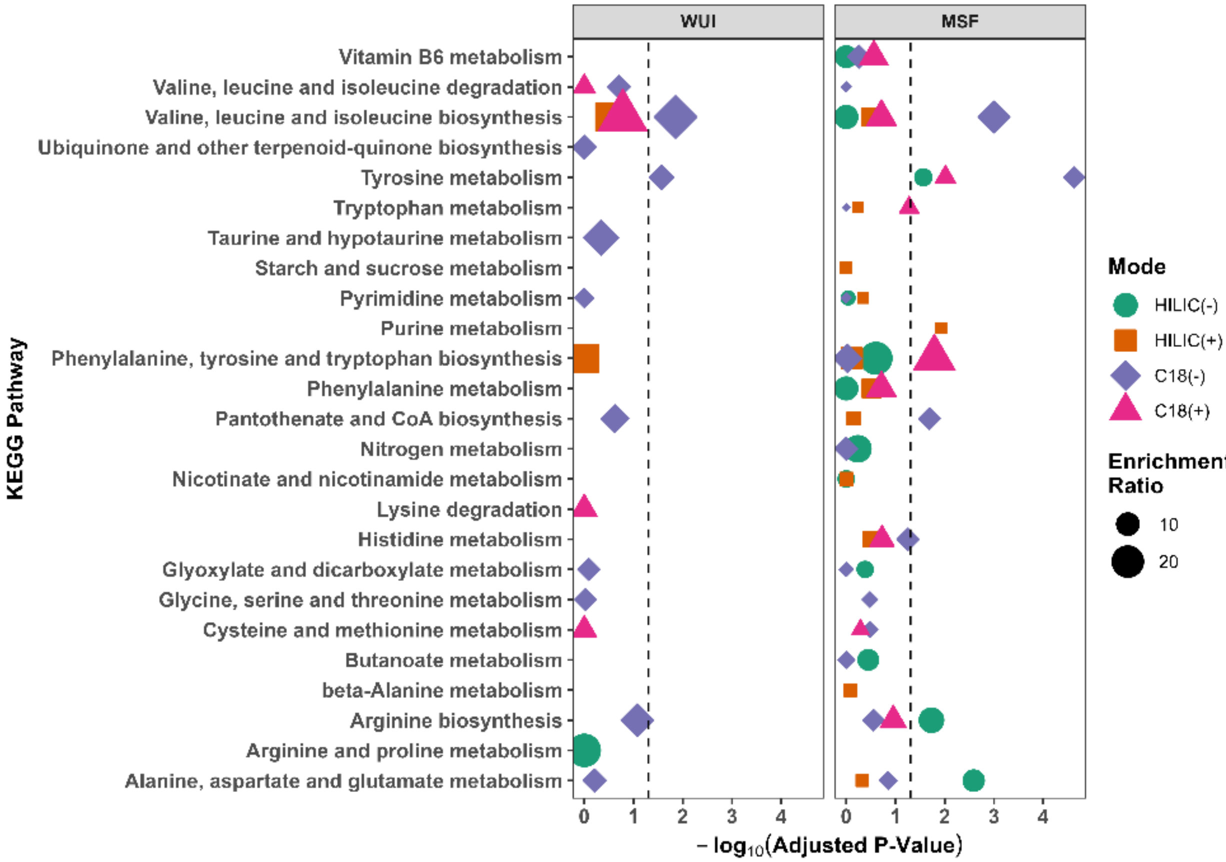


Figure 3.2: Metabolic enrichment plot for the comparison between baseline vs. post-fire urine samples among male firefighters responding to WUI fires & MSF, by four separation-ESI modes. Statistical significance was determined by Fisher's Exact test. Multiple testing was adjusted using Bonferroni's method to avoid false positives. The enrichment ratio was defined as the ratio of the number of significant hits from the user input list of differential metabolites to the number of expected metabolites in each pathway. Separation-ESI is marked in green, orange, purple, and pink. The enrichment ratio is reflected by the size of the dot where large sizes indicate more enrichment. The adjusted P-values were -log10 transformed.

3.4 Discussion

Our comparison of the urinary metabolomes of firefighters responding to WUI fires and MSF before and after fire exposure revealed both shared and unique alterations in metabolites and metabolic processes for each fire exposure. For both exposures, metabolites predominantly included endogenous molecules such as carnitine, bile acids, fatty acids, sterols, steroid hormones, as well as biomarkers of environmental chemicals such as pesticides, PFAS, phenols, and phthalates. These metabolites were involved in a wide spectrum of metabolic processes, and we observed enrichment in numerous metabolic pathways, including valine, leucine, and isoleucine biosynthesis and tyrosine metabolism, among others. Notably, valine, leucine, and isoleucine biosynthesis and tyrosine metabolism were perturbed after both types of fires. These findings suggested that like structure fires, exposure to WUI fires caused significant physiological stress in firefighters, potentially leading to long-term health implications. Additionally, while both types of fires caused metabolic disturbances, the specific pathways enriched in response to individual fires differed, likely reflecting the unique exposures of the fire events.

3.4.1 WUI fire exposure & health risks

We detected both endogenous molecules and exogenous chemicals in WUI urine samples, with most differential metabolites coming from the endogenous class. Specifically, out of the 176 differential metabolites, 11 were environmental chemicals including pesticides, PFAS, phthalates, and tobacco related compounds. Possible sources of these chemicals include ash , soot, firefighting foam and toxic smoke[3], and lifestyle factors that relate to tobacco use. Beyond chemical hazards, firefighters attending WUI fires face significant physical challenges. Heat stress, combined with physical exertion, could be another significant exposure for firefighters, as it not only intensifies muscle fatigue but also poses a risk to the cardiovascular system[84]. Therefore, the interplay between chemical and physical stressors likely contributed to the observed changes in metabolic profiles and processes, a dynamic that warrants further investigation.

In this study, we identified various metabolites whose levels were significantly altered after fire exposure. The metabolites discussed here were chosen based on their biological relevance to metabolic processes affected by oxidative stress and inflammation, as well as their statistical significance in our analysis. We focused on those that play critical roles in energy production, immune response, and oxidative stress, which are central to the physiological impact firefighters experience during fire exposure as well as the development of cancer.

3.4.2 Amino acid metabolism and oxidative stress

Two pathways were significantly enriched after both WUI and MSF responses, including valine, leucine, isoleucine biosynthesis, and tyrosine metabolism. Valine, leucine, and isoleucine (Appendix B Figure B.3) are essential amino acids (branched-chain amino acids, BCAAs) that are obtained via external sources and are crucial for protein synthesis and energy production. Firefighting is physically demanding, which may lead to increased dietary intake of BCAAs via energy drinks or nutritional supplements leading to enrichment in metabolism of essential amino acids. Persistent elevation in circulating BCAAs can lead to elevated cardiovascular risks over time[85]. Tyrosine is a non-essential amino acid that can be synthesized from phenylalanine and plays several important roles including in protein synthesis and as a precursor for neurotransmitters[86]. Disruption of tyrosine metabolism is linked to various conditions, including several types of cancer, such as gastroesophageal malignancies[87] and lung cancer[88]. Additionally, variation in tyrosine metabolism can affect the production of neurotransmitters such as dopamine and norepinephrine, which are crucial for stress responses and cognitive performance[86, 89]. We observed elevated tyrosine levels after both WUI (Appendix B Figure B.3, Figure B.4) and MSF exposures[38], likely as an acute anti-oxidative and stress response due to the beneficial effect of tyrosine on humans under acute stressors[90]. Similarly, increased tyrosine was associated with increased oxidative stress among patients with chronic diabetes-related complications[91].

The arginine biosynthesis pathway was enriched in MSF post-fire samples compared to baseline. Arginine biosynthesis was also potentially disrupted in patients with high oxidative stress[91]. Serine was elevated after WUI fire exposure (Appendix B Figure B.3). Serine contributes to cellular metabolism largely through refueling one-carbon (1C) metabolism which provides 1C units (methyl groups) that are required for methylation processes including DNA methylation and protein post-translational modification[92]. Serine also plays a vital role in maintaining metabolic homeostasis and health in stress situations[93]. Additionally, cancer cells require 1C units for high proliferation and elevated levels of serine advantages tumors and drives oncogenesis[92, 94]. Elevated level of serine post WUI fire exposure (C18 mode) may indicate cellular repair triggered by oxidative stress from fire exposure. Chronic dysregulation of serine metabolism and disruptions in one-carbon metabolism might affect genomic stability and impair various cellular functions, potentially leading to long-term health consequences such as increased cancer risk. We previously identified enrichment in glycine, serine, and threonine metabolism when comparing post-fire to baseline urine samples from MSF firefighters analyzed in HILIC and C18 mode, though it did not reach statistical significance[38]. Similarly, our re-analysis of MSF urine samples reported this pathway, but it remained statistically nonsignificant, after limiting analysis to level 1 metabolites.

Observed enrichments in both essential and non-essential amino acids likely reflect a complex set of responses to WUI fire exposures, potentially involving altered energy production and antioxidant responses in firefighters. Chronic disturbance in these critical pathways can cause elevated risks of cardiovascular conditions and increased oxidative and inflammatory burden that are closely related to cancer development and progression[68, 69], which may partially explain the elevated cancer risks of firefighters.

3.4.3 Metabolism of vitamins and lipids, and disruption of energy production, oxidative stress

Pantothenate and CoA biosynthesis were enriched after experiencing WUI fires and significantly enriched after MSF exposure. Pantothenate is a vitamin that is essential for the synthesis of coenzyme A (CoA), which is crucial for numerous metabolic processes, including the citric acid cycle for energy production, fatty acid synthesis and oxidation, and lipid synthesis. Upregulation of CoA also occurs in response to cellular oxidative stress[95].

Ubiquinone (coenzyme Q) is a crucial component for electron transport and ATP generation whose deficiency may cause conditions such as type 2 diabetes and cardiovascular disease[96, 97]. Terpenoid-quinones are derived from terpenoids and quinones, which are important for electron transport and protection against oxidant stress[98, 99]. Homogentisate is an integral compound in the ubiquinone and other terpenoid-quinone biosynthesis process and was elevated postfire (Appendix B Figure B.3), possibly indicative of cellular repair induced by oxidative stress from WUI fire exposures. Chronic exposure and metabolic perturbation could lead to impairment in energy production and overall elevated risk of cancer due to oxidative stress among firefighters.

Overall, the enrichment patterns observed indicated significant metabolic disturbances following WUI fire exposure. These changes reflect increased physical and physiological stress, leading to disruptions in metabolism of amino acids, lipids, and vitamins, which are likely related to energy production and oxidative response due to firefighting. Chronic disruptions of these metabolic processes can result in an elevated risk of cardiovascular and neurological conditions, oxidative and inflammatory burdens, genomic instability, and cancer[68, 69, 85, 89]. A case-control study of untargeted metabolomics in breast cancer reported that pre-diagnostic disturbances in several metabolomic pathways were predictive of breast cancer up to 22 years later[22]. Several of these were pathways that we report here in firefighters, including tyrosine metabolism, tryptophan metabolism, valine, leucine, and isoleucine biosynthesis, and ubiquinone and other terpenoid-quinone biosynthesis. This observation further supports the conclusion that firefighters face increased cancer risks.

3.4.4 WUI-MSF comparison

We detected markers for endogenous and exogenous chemicals in MSF urine samples. Out of a total of 652 differential metabolites, 32 were environmental chemicals. Overall, MSF exposure resulted in a greater number of differential metabolites compared to WUI fires. Consequently, a wider range of metabolic processes were found to be enriched.

In addition to the shared enrichment with WUI, MSF exposure introduced a broader set of disturbances to metabolic processes including metabolism of purine, arginine, aspartate, glutamate, alanine, phenylalanine, and tryptophan. Purines are critical to nucleic acid synthesis[100]. Arginine plays an important role in detoxification of ammonia[101] and has been associated with increased oxidative stress[91]. Aspartate regulates nucleotide synthesis and serves as a precursor for four essential amino acids[102]. Glutamate is the most abundant excitatory neurotransmitter in the central nervous system (CNS) and is involved in various cognitive functions[103]. L-alanine plays various important roles in the body including in protein synthesis and the functioning of the immune system, as well as an energy source for intense exercise[104]. Phenylalanine is a building block of other amino acids, and a precursor of tyrosine and dopamine[105]. The enrichment of phenylalanine and tyrosine metabolism, indicative of an anti-oxidative response to fire-related tasks and chronic stress, may lead to cognitive impairment and an increased risk of several types of cancers[87, 88]. Tryptophan is an essential amino acid that serves as a precursor for serotonin and the kynurenine pathway, which are crucial for mood regulation, immune response, and cognitive function[60]. MSF postfire samples had elevated levels of tryptophan, kynurenic acid, and serotonin. We previously reported increased levels of tryptophan and tryptophan derivatives such as kynurenic acid after structure fires[38]. Other studies have also reported altered expression of tryptophan associated with air pollution, which may introduce the same stressors as those experienced by MSF firefighters during structure fire tasks[61]. The pathways disrupted by MSF exposure indicate potential oxidative stress and disturbances in cognitive and genetic functions. Chronic disruption of these critical pathways can pose a wide range of health risks, including cognitive impairment, genetic instability, and a heightened overall risk of cancer.

The number of shared differential metabolites following WUI fires compared to MSF alone indicates both common and unique metabolic perturbations. Both WUI and MSF exposure induced changes in valine, leucine, isoleucine, and tyrosine, demonstrating shared metabolic perturbations in metabolism of amino acids. WUI fires affected pathways related to valine, leucine, isoleucine, and tyrosine biosynthesis while MSF uniquely impacted the metabolism or biosynthesis of purine, phenylalanine, tryptophan, arginine, alanine, aspartate, and glutamate. The WUI pathways suggest impacts on energy production and oxidative stress, whereas the higher number of differential metabolites and unique pathways from MSF suggest a more pronounced disruption of neurotransmitters and DNA/RNA synthesis, in addition to altered energy production and oxidative stress. Both types of fires may present significant cancer risks due to perturbations in critical metabolic processes. Overall, MSF resulted in more extensive alterations in both individual metabolites and metabolic processes compared to WUI fires, which may contribute to a wider range of health concerns. Longitudinal or targeted studies are needed to evaluate the associations of urine metabolites with changes in other markers of cancer risk, such as epigenetic changes, and ultimately cancer incidence.

Chapter 4. Evaluating Urine Metabolic Profiles with Training Fire Exposure Among Female Firefighters

Abstract

Female firefighters face serious health risks including elevated risk for cancer and reproductive conditions, although underlying metabolic mechanism(s) are not fully understood. This study aimed to identify urinary metabolites and metabolic functions associated with training fire exposure among female municipal firefighters. High-resolution metabolomics (HRM) was applied to urine sample collected at baseline and after live-fire burn room/tower or flashover training fire exposure from female firefighters in the Fire Fighter Cancer Cohort Study (FFCCS). To identify differentially expressed metabolites (DEMs), differential analysis was performed using linear mixed-effects models adjusting for demographic confounders including age, socioeconomic factors, cancer history, dietary and medication behaviors, with false discovery rate adjustment. Functional enrichment analysis (FEA) was carried out using metabolite-set enrichment analysis (MSEA) from MetaboAnalyst to identify enriched metabolic processes. A secondary stratified analysis was carried out to investigate the effect of training fire type on metabolome after fire exposure using a linear regression model while adjusting for covariates. One hundred female firefighters participated, resulting in a total of 200 urine samples (100 baseline, 100 postfire). The 200 samples underwent HRM analysis in four separation-ionization modes including HILIC(+), HILIC(-), C18(+), and C18(-), annotating against an in-house library of ~1200 standards. We identified 200, 300, 280, and 306 metabolites and 10, 9, 23, and 19 post-training fire DEMs from the four modes, respectively. The FEA process identified that glycerophospholipid metabolism was significantly enriched at a p-value 0.05 level. Stratified analysis identified a total of 17 DEMs by fire type and increased relative ion intensities across all DEMs during burn room/tower fires compared to flashover fires. Female firefighters exposed to training fires exhibited a set of metabolic changes, particularly related to cellular damage from oxidative stress. These observations suggest a potential pathway for chronic inflammation with long-term fire exposure, which may help explain the higher prevalence of certain health

conditions observed in female firefighters. Increased intensity DEMs were found following burn room/tower as compared with flashover fires.

4.1 Introduction

Firefighters are exposed to known or probable carcinogens[26], including but not limited to polycyclic aromatic hydrocarbons (PAHs)[13], benzene[13], formaldehyde[27], and per-and polyfluoroalkyl substances (PFAS)[106]. As a result, they experience a higher risk for select cancers, such as skin melanoma[9], lung[26], leukemia[26], kidney[29] and prostate cancer[9, 29, 30], according to study partnered with firefighters in Florida[9, 11], Washington[31], California[29, 32], and other US cities[10, 26, 33] where firefighters in the United States (US) are reported to face excess overall cancer mortality (SMR=1.14; 95% CI 1.10-1.18) as compared to the general US population[1, 10]. In addition, the International Agency for Research on Cancer (IARC) recently classified firefighters' occupational exposure as carcinogenic to humans with sufficient evidence for fireground exposures and bladder cancer[1]. Despite increasing studies on the health effects of firefighting, evidence on its impact among female firefighters remains sparse and evidence is mixed. Some studies report that female firefighters had significantly increased bladder (SMR=33.51; 4.06 -121.05, sample size < 5), brain (aOR=2.54; 1.19-5.42), and thyroid (aOR=2.42; 1.56-3.74) cancer risk[9-11]. However, a study on mortality and cancer incidence among Australian female firefighters showed a similar cancer rate compared with the general population[12].

The relationship between fireground exposure and adverse health effects in female firefighters is not well understood. In addition to elevated cancer risks, adverse exposure may also contribute to reproductive issues in female firefighters. Previous epidemiological studies have shown that female firefighters have high rates of adverse reproductive outcomes, including miscarriage[15], preterm birth[15], and lower levels of anti-müllerian hormone (AMH)[107], an important diagnostic measure directly associated with reproductive reserve. However, the link between key toxicants disturbed metabolisms and adverse health outcomes is yet to be established. This will enable a mechanistic view on the exposure-disease relationship and effective development of preventative measures to protect female firefighters from hazardous occupational exposures. Our previous study in men firefighters identified a large set of DEMs comparing metabolites in urine samples before and after municipal structure fire (MSF) exposure[38]. We thus hypothesize that there will be DEMs by fire exposure in female firefighters, given their exposure resembles that of men firefighters, although the specific metabolites may vary due to biological and behavioral variation by sex.

Firefighters participated in live-fire training that involved exposure to flashover and burn tower/building fires. Typically, firefighters remain stationary during flashover training whereas they actively perform a search or rescue task during burn room/tower simulations. While research on the metabolic effects of different fire types remains limited, the exposure conditions varied significantly between flashover and burn room training due to differences in burning materials and the activity status.

Given the notable lack of comprehensive research on fire-related metabolic changes in female firefighters, our study aimed to analyze changes in urine metabolic profiles before and after exposure to training fires that simulate municipal structure fires (MSFs). We expect to identify distinct DEMs specific to female firefighters following fire exposure, with some overlap in DEMs between female and men firefighters responding to MSFs. We further anticipated discovering fire type-specific DEMs among female firefighters, and that these metabolic changes are expected to have important implications for firefighter health.

4.2 Methods

4.2.1 Study population and sample collection

Building on existing Fire Fighter Cancer Cohort Study (FFCCS) framework[108], this study enrolled both career and volunteer female firefighters across multiple fire departments and collected urine sample pre and post fire exposure from live-training fires. All baseline and post-fire urine samples were collected from October 2022 to July 2024.

4.2.2 Sample preparation

All postfire samples were collected within 1.8-11.0 hours after baseline. Urine samples were then transported on ice to the University of Arizona. After collection in 150 mL plastic collection cups, urine samples were stored on dry ice and shipped priority overnight to the University of Arizona for processing. Upon receipt, samples were stored at -80°C until processed. Urine was thawed in a rotary incubator at 25°C for two hours or until fully thawed. Samples were mixed via inversion, then divided into 2 mL and 12 mL aliquots and stored in plastic cryovials. During sample processing, specific gravity was measured using an Atago pocket refractometer. Processed samples were then stored at -80°C.

4.2.3 High-resolution metabolomics

All urine samples were sent on ice to the Comprehensive Laboratory for Untargeted Exposome Science at Emory University for high-resolution metabolic profiling. Urine samples were prepared in batches of up to 84 study samples containing up to 12 quality assurance-quality control (QAQC) samples using automated liquid handling (Opentron OT2) and 96-well plates. Prior to analysis, samples were thawed at 4ºC, and 30 µL urine was extracted by adding 90 µL acetonitrile containing 13C labeled internal standards (ISs). Treated samples were vortexed for 2 minutes, equilibrated at 4°C for 30 min, and then centrifuged for 45 min at 3,220×g, at 4ºC. Following centrifugation, two 30 µL aliquots of the supernatant were transferred to 96-well plates, each containing either 60 µL of water (for Reverse Phase Chromatography (C18)) or 60 µL of a 1:1 acetonitrile/water so- lution (for Hydrophilic Interaction Liquid Chromatography (HILIC)). These were vortexed for another 2 minutes and stored in a refrigerated autosampler until they were analyzed.

Targeted High-Resolution Metabolomics (HRM) was accomplished using two separate systems configured for C18 or HILIC analysis that included a Vanquish Duo Ultra Performance Liquid Chromatography (Thermo Fisher Scientific, Rockford, IL, USA) coupled to an Exploris120 HRMS system (Thermo Fisher Scientific, Rockford, IL, USA). LC column temperatures were set to 40°C (HILIC) and 30°C (C18), and the automatic sampler temperature was set at 5°C. Samples were analyzed using dual column chromatography with mobile phases optimized for positive or negative ionization. All samples were analyzed with reverse phase Higgins TARGA C18 5µm 50x2.1mm column (Higgins Analytical, Inc, Mountain View, CA, USA) in both positive mode and negative mode. HILIC chromatography separation was accomplished using a SeQuant ZIC-HILIC 3.5µm 50x4.6mm column (Merck KGaA, Darmstadt, Germany) for positive mode and a XBridge Amide 3.5µm 3.0x50mm column for negative mode. The mobile phase for the reverse phase C18 analysis included water containing 0.1% formic acid (B) and acetonitrile containing 0.1% formic acid (A) for positive mode; and 10mM ammonium acetate in water (B) and 97.5/2.5 (v/v) acetonitrile/water (A) for negative mode; HILIC included water containing 0.1% formic acid (B) and acetonitrile containing 0.1% formic acid (A) for positive mode; and 10mM ammonium acetate in water adjusted to pH 9.5 (B) and 97.5/2.5 (v/v) acetonitrile/water (A) for negative mode Flow rates ranged from 0.3 mL/min to 0.6 mL/min, and the total run time was 7.5 min.

4.2.4 Feature preprocessing and metabolite annotation

Following analysis of study and QAQC samples, raw instrument files were converted to mzML with peak picking and extracted using XCMS. Detected m/z were grouped using RamClustR to identify m/z's corresponding to the same compound. Batch correction was performed using log2-transformed raw intensities with WaveICA 1.0, which corrects for both inter- and intra-batch effects.

Metabolites were identified by comparing detected m/z and retention time to a database of 1,200 standards analyzed using the same method parameters that included a wide range of environmental and endogenous compounds. Metabolite identifications were achieved uniquely by matching m/z and retention time with a tolerance of 5ppm and 15 seconds, respectively.

4.2.5 Data preprocessing and statistical analysis

We first describe demographic characteristics for participants. For differential metabolomic analysis, we restricted all analyses to metabolites annotated with level 1 annotation confidence and present in at least 75% of the samples. All metabolic features' ion intensities were log2 transformed and standardized to stabilize variation and facilitate other linear model assumptions. Differential analysis was performed using a linear mixed effects model fitted on the relative intensity of each metabolite identified with exposure status being the major predictor while adjusting for important demographic covariates including age, education, Hispanic ethnicity, and type of live fires. The model slope was then extracted from the modeling results and interpreted as changes in the expression of metabolite with fire exposure where positive slope indicated increased expression while negative one indicated decreased expression. P-value was also extracted from the model output and multiple testing was adjusted by controlling the FDR at 0.05 level. We identified DEMs as those with a p-value smaller than 0.05. A volcano plot was made to showcase the overall differential status of the metabolome, with identified DEMs highlighted. For easy comparison with previous studies, fold changes were also calculated and presented in a circular bar chart for DEMs comparing postfire and baseline means to showcase the changes at a metabolite-level, together with a two-sample t-test. For metabolites present in less than 75% of our samples, we performed a two-sample t-test for each to salvage important metabolic signals and complement the main analysis.

Additionally, we performed a secondary analysis to compare female firefighters' postfire metabolic profiles by fire type. A multiple linear regression model was fitted on preprocessed intensities of each metabolite with fire type being the main predictor while adjusting for covariates including Hispanic ethnicity, age, rank, and other important demographics. P-value was extracted, and multiple testing was adjusted for with a threshold for FDR $q< 0.05$.

All statistical analyses were performed in the R programming environment (version 4.4.1)[48].

4.2.6 Functional enrichment analysis

Following preprocessing and differential analysis, we performed FEA to investigate the difference in metabolic profiles by live-fire exposure at the biological pathway level. Targeted quantitative enrichment analysis was performed using MetaboAnalyst (version 6.0)[46, 83]. Sum normalization and auto-scaling were applied to remove systematic variability. Only pathways with at least 2 entries were included. We defined the statistical significance for enrichment as a p-value of 0.05. Multiple testing was adjusted by controlling the FDR at 0.05 level. We only interpreted significantly enriched metabolism(s).

4.3 Results

4.3.1 Study population and sample

This analysis included 100 female firefighters and their urine sample from baseline and after postfire exposure. The median (min, max) duration in hours between baseline samples and postfire samples was 7.5 (2.0, 11.0) hours. We sent a total of 200 urine samples, 2 for each firefighters, for targeted metabolomics analysis and got back data on 4 LC-ionization modes including HILIC(-), HILIC(+), C18(-), and C18(+).

Among the 100 female firefighters, 45% held ranks as career firefighters with the remaining serving as volunteers, 31% were Hispanic, 61% had a college degree, and 21% had a family cancer history. Roughly half of the participants responded to flash-over training fires and the other half to burn room/tower burn training fires. (Table 4.1).

Table 4.1: Summary statistics of demographics for female firefighters responding to training fires (N=100) for the female firefighter study.

	N = 100[1]
Age	30.0 (21.0, 40.0)
Hispanic ethnicity	31 / 100 (31%)
Education	
Some Colleges or lower	39 / 100 (39%)
College graduate	61 / 100 (61%)
Type of fire	
Burn Room/Tower Burn	49 / 100 (49%)
Flash Over	51 / 100 (51%)
Days had at least one drink in past week	
>2	11 / 100 (11%)
0	39 / 100 (39%)
1	38 / 100 (38%)
2	12 / 100 (12%)
Duration between baseline and postfire	7.5 (2.0, 11.0)
(Missing)	7

[1]Median (IQR) or Frequency (%).

4.3.2 High-resolution metabolomics

We identified a total of 247, 202, 321, and 291 urinary metabolites with level 1 annotation confidence from HILIC(-), HILIC(+), C18(-), C18(+) mode, respectively. Most of the identified metabolites were endogenous with a small proportion of them being markers for per- and polyfluoroalkyl substances (PFAS), pesticides, phenol, phthalates, and cigarette use. After removing metabolites that were present in less than 75% of samples, a total of 224, 180, 300, and 263 features were retained from HILIC(-), HILIC(+), C18(-), C18(+) mode, respectively.

4.3.3 Differential analysis and statistical analysis

After adjustment for age, rank, education, Hispanic ethnicity, fire type, since baseline fires, the main model identified 10, 9, 23, and 19 DEMs by fire exposure for HILIC(+), HILIC(-), C18(+), and C18(-) mode, respectively, when comparing baseline and postfire urine samples of female firefighters responding to training fires (Table 4.2). Figure 4.1 presents the overall differential status with top 10 DEMs annotations for each of the separation-ionization mode. Overall, we saw more upregulations than downregulations after training fire exposure in female firefighters, like what we observed in men firefighters.

Table 4.2: DEMs by statistical significance within each separation (ionization) mode comparing post-fire to baseline urine samples for female firefighters responding to training fire exposure. MZ: mass to charge ratio; RT: retention time in seconds; Slope: the coefficient estimates for the exposure status (baseline-postfire: 0-1) term of the main model. Positive slopes indicated an increase in the relative ion intensity of metabolites after fire exposure whereas negative slopes indicated a decrease.

Metabolite	Molecular Formula	PubChem CID	MZ	RT	Adduct	Slope	P-value	Mode
Nicotine	C10H14N2	942	163.12	333.80	M+H	0.38	0.01	HILIC(+)
Valine	C5H11NO2	6287	118.09	263.40	M+H	0.17	0.01	HILIC(+)
Fenobucarb	C12H17NO2	19588	208.13	67.70	M+H	0.32	0.02	HILIC(+)
4-Quinolinecarboxylate	C10H7NO2	10243	174.06	94.30	M+H	1.14	0.02	HILIC(+)
2,4-Dihydroxypteridine	C6H4N4O2	10250	165.04	97.40	M+H	1.51	0.02	HILIC(+)
Guanidinoacetate	C3H7N3O2	763	118.06	306.90	M+H	0.53	0.03	HILIC(+)
N-Acetylphenylalanine	C11H13NO3	74839	249.12	73.20	M+ACN +H	0.63	0.04	HILIC(+)
L-Carnitine	C7H15NO3	10917	162.11	294.30	M+H	0.37	0.04	HILIC(+)
L-Cartinine	C7H15NO3	2724480	162.11	297.10	M+H	0.37	0.04	HILIC(+)
Sphinganine	C18H39NO2	91486	302.31	203.50	M+H	-0.07	0.05	HILIC(+)
Succinic acid	C4H6O4	1110	117.02	306.50	M-H	0.19	0.03	HILIC(-)
[C8.1]-2-Octenoic acid	C8H14O2	5282713	141.09	68.00	M-H	0.33	0.03	HILIC(-)
Benzoate	C7H6O2	243	121.03	82.02	M-H	0.41	0.04	HILIC(-)
4-Hydroxybenzaldehyde	C7H6O2	126	121.03	67.70	M-H	0.41	0.04	HILIC(-)
Benzoic acid	C7H6O2	243	121.03	70.40	M-H	0.41	0.04	HILIC(-)
Methyl Vanillate	C9H10O4	19844	181.05	70.69	M-H	0.44	0.04	HILIC(-)
4-Hydroxyphenylpyruvic acid	C9H8O4	979	179.04	70.60	M-H	0.64	0.04	HILIC(-)
3-Hydroxybenzaldehyde	C7H6O2	101	121.03	64.03	M-H	0.45	0.04	HILIC(-)
Alpha-ketoisovaleric acid	C5H8O3	49	231.09	69.00	M-H	1.44	0.05	HILIC(-)
7-Ketochenodeoxycholate	C24H38O4	53477693	391.28	270.17	M+H	0.19	0.00	C18(+)
Valine	C5H11NO2	6287	118.09	21.60	M+H	0.20	0.01	C18(+)
5-Aminovaleric acid	C5H11NO2	138	118.09	19.48	M+H	0.20	0.01	C18(+)
Deoxycarnitine	C7H15NO2	725	146.12	22.37	M+H	0.79	0.02	C18(+)
Acetylcholine	C7H16NO2+	187	146.12	20.31	M+	0.79	0.02	C18(+)
N, N-Dimethyl-1,4-Phenylenediamine	C8H12N2	7472	137.11	22.97	M+H	0.82	0.02	C18(+)
Mannitol	C6H14O6	6251	205.07	21.41	M+Na	1.16	0.02	C18(+)
Histamine	C5H9N3	774	112.09	17.45	M+H	0.84	0.02	C18(+)

Metabolite	Molecular Formula	PubChem CID	MZ	RT	Adduct	Slope	P-value	Mode
N-acetyl-tyrosine	C11H13NO4	68310	224.09	44.85	M+H	0.88	0.02	C18(+)
Glycochenodeoxycholic acid	C26H43NO5	12544	450.32	255.50	M+H	0.83	0.02	C18(+)
O-Acetylcarnitine	C9H17NO4	439756	204.12	23.00	M+H	0.77	0.03	C18(+)
Acetyl-DL-carnitine	C9H17NO4	7045767	204.12	19.97	M+H	0.77	0.03	C18(+)
Salsolinol	C10H13NO2	91588	180.10	21.41	M+H	0.47	0.03	C18(+)
Dihydrouracil	C4H6N2O2	649	132.08	22.29	M+NH4	1.09	0.03	C18(+)
Creatine	C4H9N3O2	586	132.08	22.29	M+H	1.09	0.03	C18(+)
Pterin	C3H6N2S	135398660	164.06	23.74	M+H	1.21	0.03	C18(+)
Testosterone	C19H28O2	6013	289.22	262.32	M+H	0.86	0.04	C18(+)
Aminoisobutanoate	C4H9NO2	64956	104.07	21.30	M+H	0.19	0.04	C18(+)
Gamma-Aminobutyrate	C4H9NO2	119	104.07	20.73	M+H	0.19	0.04	C18(+)
Alpha-aminobutyric acid	C4H9NO2	80283	104.07	19.72	M+H	0.19	0.04	C18(+)
2,6-Dihydroxypyridine	C5H5NO2	69371	112.04	25.93	M+H	0.40	0.04	C18(+)
Propionyl-L-carnitine	C10H19NO4	107738	218.14	21.55	M+H	0.63	0.04	C18(+)
Propanoylcarnitine	C10H19NO4	188824	218.14	22.26	M+H	0.63	0.04	C18(+)
[C10.1]-Cis-2-Decenoic acid	C10H18O2	5356596	169.12	237.80	M-H	0.20	0.01	C18(-)
2’,4’-Dihydroxyacetophenone	C8H8O3	6990	151.04	191.70	M-H	0.19	0.01	C18(-)
Resorcinol Monoacetate	C8H8O3	5055	151.04	190.10	M-H	0.19	0.01	C18(-)
[C19.1]-10Z-Nonadecenoic acid	C19H36O2	5312513	295.26	397.20	M-H	0.21	0.01	C18(-)
4-Hydroxybenzoate; 4-Hydroxybenzoic acid	C7H6O3	135	137.02	21.00	M-H	0.44	0.02	C18(-)
[C18.2]-11-Octadecen-9-ynoic acid	C18H30O2	5312688	277.22	325.00	M-H	0.49	0.02	C18(-)
3-(4-hydroxyphenyl) propanoic acid	C9H10O3	10394	165.06	20.00	M-H	0.39	0.03	C18(-)
N-Acetylglucosamine	C8H15NO6	439174	256.06	20.90	M+Cl	0.26	0.03	C18(-)
N-Acetylglutamate	C7H11NO5	70914	188.06	18.00	M-H	0.25	0.03	C18(-)
[C12.1]-5-Dodecenoic acid	C12H22O2	5312378	197.15	263.30	M-H	0.08	0.03	C18(-)
Alpha-Muricholic acid	C24H40O5	53477700	407.28	208.30	M-H	0.82	0.04	C18(-)
Ursocholic acid	C24H40O5	122340	407.28	199.30	M-H	0.82	0.04	C18(-)
4-Aminobenzoate	C7H7NO2	978	136.04	20.60	M-H	0.20	0.04	C18(-)
2-Deoxy-D-Glucose	C6H12O5	439268	163.06	20.70	M-H	0.25	0.04	C18(-)
Porphobilinogen	C10H14N2O4	1021	225.09	19.30	M-H	0.72	0.04	C18(-)
Glutamate	C5H9NO4	33032	146.05	18.60	M-H	0.29	0.04	C18(-)
2-Keto-3-Deoxy-D-Gluconic Acid	C6H10O6	194024	177.04	19.00	M-H	0.19	0.04	C18(-)
Theophylline	C7H8N4O2	2153	179.06	34.50	M-H	1.28	0.04	C18(-)
2,6-Dihydroxypyridine	C5H5NO2	69371	110.02	19.80	M-H	0.43	0.05	C18(-)

4.3.4 Functional enrichment analysis

Metabolite-set enrichment analysis (MSEA) by mapping all DEMs to KEGG pathways revealed no pathways that were enriched at an FDR<0.05, and 1 significantly enriched metabolism at a relaxed p-value< 0.05: glycerophospholipid metabolism. Enrichment status from each of the 4 modes was presented in Figure 4.1C. At metabolite level, the only hit from our identified DEMs in the glycerophospholipid metabolism was acetylcholine (slope=0.79, p-value=0.02). By the hard threshold of p-value 0.05 or FDR 0.05, no other significant enrichments were observed in this study, though other metabolisms may still be worth investigating given their important roles in biological processes.

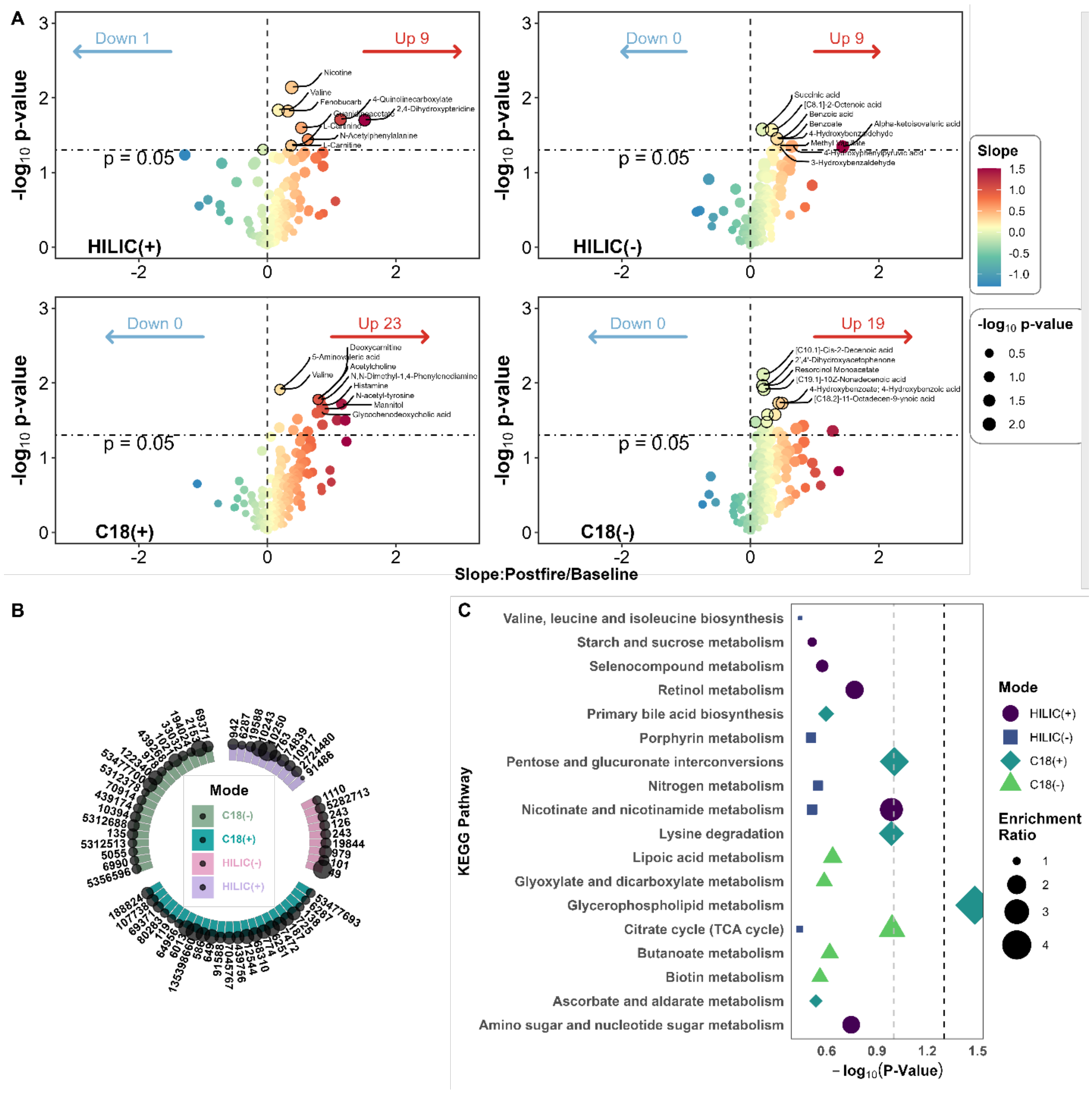


Figure 4.1: Combo plot for differential analysis and functional enrichment analysis. **A** Volcano plot of DEMs comparing post-fire to baseline urine samples from female firefighters responding to training fires. The slope term was from the main model where exposure status (postfire/baseline) was the main predictor and preprocessed ion intensity was the response. Upregulation is marked in red whereas downregulation in light blue. The horizontal dashed line marked "p-value=0.05" is the boundary that served as the threshold for differential status in this

study. P-values were -log10 transformed. **B** Circular bar chart for fold changes of DEMs identified by DA. Unique CID for each of the DEMs was used for visualization purposes and a reference to metabolite name can be found in Table 2. Fold change is reflected by the sizes of the circles and mode is indicated in 4 colors. **C** Enrichment plot for the comparison between post-fire vs. baseline urine samples among female firefighters responding to training fires, by four separation(ionization) modes. Statistical significance was determined by MSEA. The vertical dashed line in black and grey marked "p-value=0.05" and “p-value=0.1”, respectively, is the boundaries that served as the thresholds for enrichment status in this study. Multiple testing was adjusted with a threshold FDR at 0.05 to avoid false positives. The enrichment ratio was defined as the ratio of the number of significant hits from the user input list of differential metabolites to the number of expected metabolites in each pathway. Separation- ionization is marked in purple, blue, dark green, and light green. The enrichment ratio is reflected by the size of the dot where large sizes indicate larger enrichment. The p-values were -log10 transformed.

4.3.5 Sub-analysis: effect of fire types

After adjusting Hispanic ethnicity, family cancer history, age, education, alcohol and marijuana use, sub-analysis on the effects of fire types (burn room/tower vs. flashover) on the metabolome using multiple linear regression identified a total of 17 DEMs by fire type, including 6, 2, 7, and 2 DEMs, by a p-value 0.05 threshold, from HILIC(+), HILIC(-), C18(+), and C18(-) mode, respectively. No significant DEMs were identified at FDR 0.05 level. A direct comparison of the relative ion intensity between burn tower/burn room fires and flash over fires was plotted using boxplots for each of the DEMs identified by the secondary model in Figure 4.2C. All DEMs by fire type saw increased ion intensities in Burn Room/Burn Tower fires than in flash over fires. Only fuberidazole increased significantly at FDR 0.05 level. A complete list of DEMs for sub-analysis with model details can be found in Supplementary Table 4. Burn room/tower fires introduced a larger number of DEMs, and thus a more intense differential profile than flashover fires in female firefighters (Figure 4.2A, B).

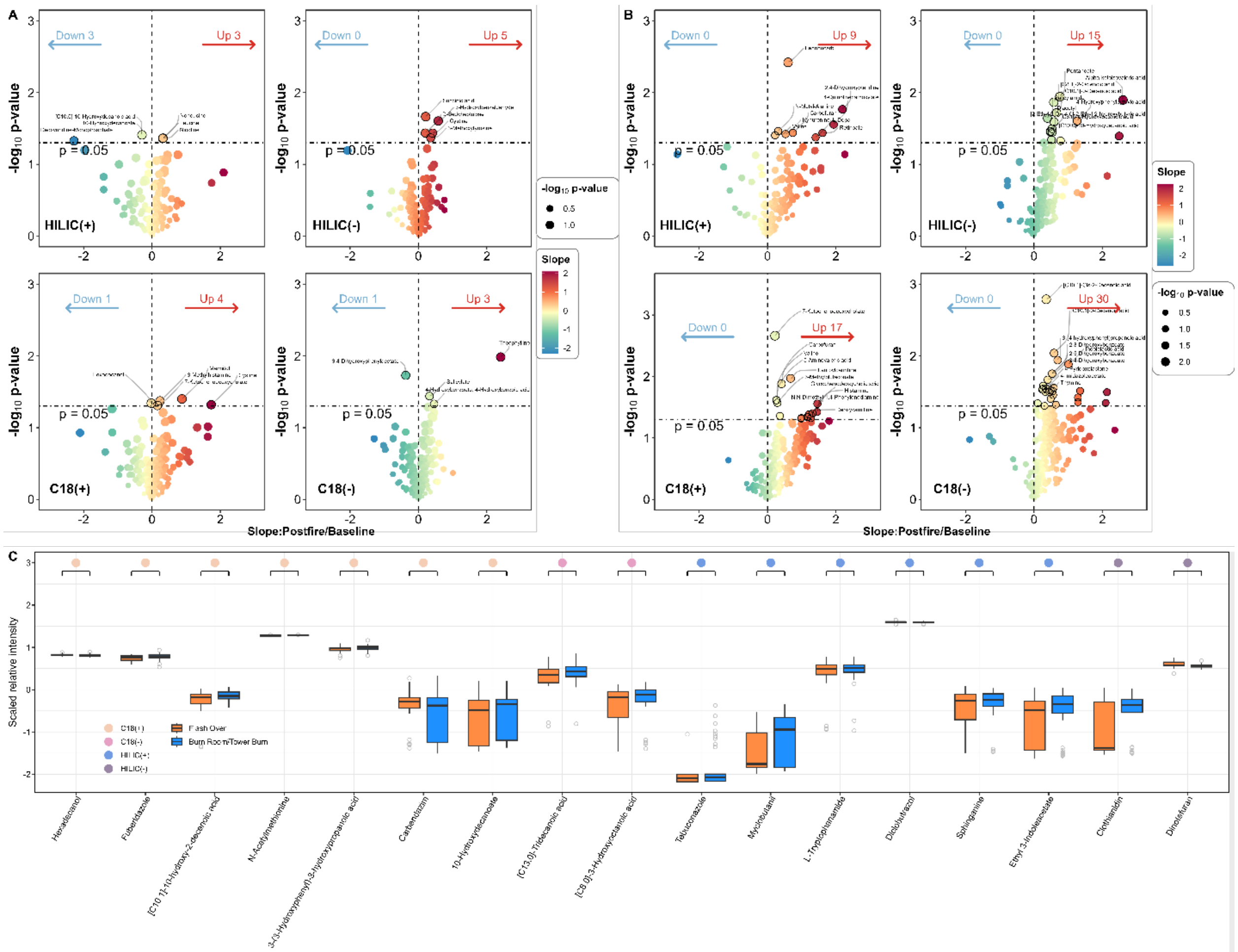


Figure 4.2: A Volcano plot of DEMs comparing post-fire to baseline urine samples from female firefighters responding to flashover training fires. The slope term was from the main model where exposure status (postfire/baseline) was the main predictor and preprocessed ion intensity was the response. Upregulation is marked in red whereas downregulation in light blue. The horizontal dashed line marked "p-value=0.05" is the boundary that served as the threshold for differential status in this study. P-values were -log10 transformed. **B** Volcano plot of DEMs comparing post-fire to baseline urine samples from female firefighters responding to burn room/tower training fires. The slope term was from the main model where exposure status (postfire/baseline) was the main predictor and preprocessed ion intensity was the response. Upregulation is marked in red whereas downregulation in light blue. The horizontal dashed line marked "p-value=0.05" is the boundary that served as the threshold for differential status in this

study. P-values were -log10 transformed. **C** Boxplots comparing metabolites that were differentially expressed at p-value 0.05 level by training fire types, organized by separation(ionization) mode. Two sample t-tests were performed to derive statistical significance. Yellow boxes represented flash-over fires while blue boxes indicated burn room/tower burn fires. Dots on the top indicated the separation(ionization) mode with orange, pink, blue, and purple representing C18(+), C18(-), HILIC(+), and HILIC(-), respectively.

4.4 Discussion

In this study, we performed targeted metabolomics to increase the understanding of health risk imposed by fire exposures in female firefighters. This approach allowed us to profile the metabolome and identify differential expressions across 100 female firefighters. Out of identified urinary metabolites, we identified 10, 9, 23, and 19 DEMs by fire exposure from HILIC(+), HILIC(-), C18(+), and C18(-) mode, respectively. These differences were enriched for glycerophospholipid metabolism. In addition, the post-fire exposure metabolomic profiles vary by fire types, suggesting metabolic differences may be strongly related to type of training fire and activities at the fire scene. Identification of DEMs and enrichments in the biological processes may help explain the increased cancer risk by fire exposure among female firefighters.

4.4.1 Training fire related enrichment in metabolism

Firefighters are exposed to high levels of oxidative stress due to inhaling smoke which includes toxic chemicals and particulate matter[109, 110]. Oxidative stress burden and chronic inflammation are related to multiple disorders, including but not limited to cancer[111], cardiovascular diseases[111], and renal disease resulting from the excessive accumulation of reactive free radicals[112]. Additionally, oxidative stress, chronic inflammation, and cancer are closely related. Long term oxidative stress can lead to chronic inflammation, which in turn leads to tumorigenesis, tumor cell survival, proliferation, and more[69].

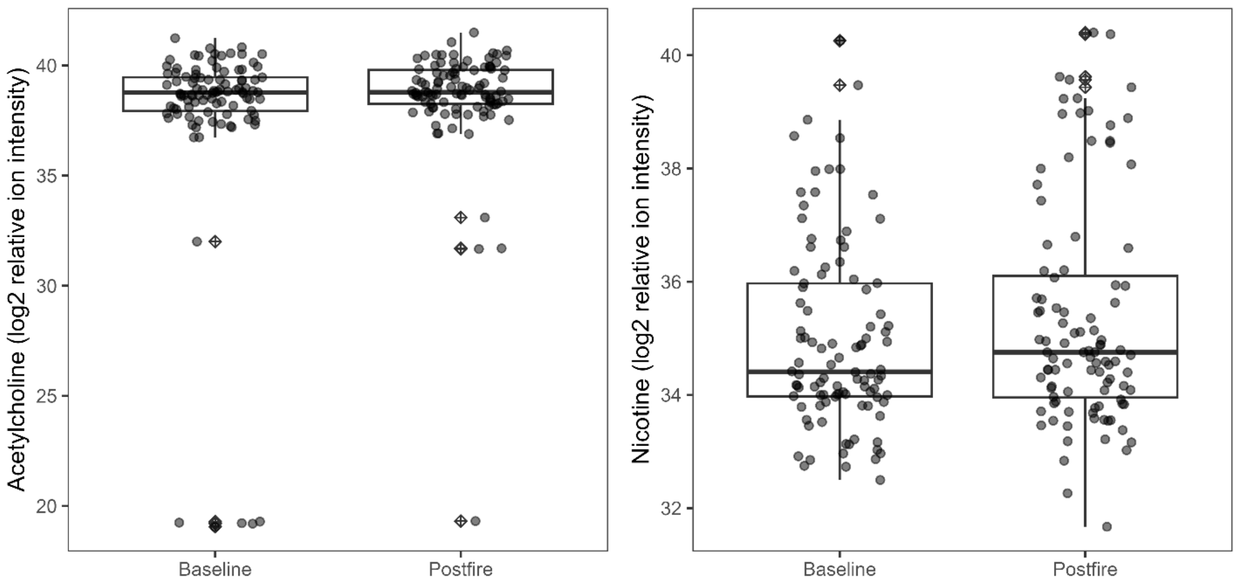


Figure 4.3: Boxplot of metabolite expression at baseline and post-exposure. Ion intensities were log2 transformed. Solid dots represent individual measurements from participants where outliers are marked in a different shape.

Existing evidence has implicated that oxidative stress alters cellular lipid metabolism[113]. Glycerophospholipids are major components of cell membranes, maintaining membrane integrity, fluidity, and are involved in signaling processes[114]. Oxidative stress can damage cellular membranes, prompting increased turnover and repair via enhanced glycerophospholipid metabolism[115].

The increased activity in glycerophospholipid metabolism may represent an adaptive response to counteract damage induced by fire exposure. The body ramps up lipid turnover and repair mechanisms to restore normal cellular functions. While this may initially be protective, chronic or repeated exposure, and the resulting persistent metabolic changes, might contribute to persistent cellular stress. Over time, such changes could potentially be linked to an increased risk of inflammatory conditions[69], cardiovascular disease[116], or cancer[68, 69] observed at higher rates in firefighters.

The hit from our identified DEMs with the metabolite set in glycerophospholipid metabolism was acetylcholine. Choline is the precursor for acetylcholine, a neurotransmitter synthesized by the enzyme choline acetyltransferase and a product of glycerophospholipid metabolism[47]. An increase in glycerophospholipid turnover can elevate the availability of choline, thereby potentially increasing acetylcholine synthesis. We saw a slightly higher level of acetylcholine after fire exposure (Figure 4.3), which might be indicative of activated glycerophospholipid metabolism in response to cellular damage from oxidative stress caused by fire exposure.

Out of the DEMs identified in this study, some display properties related to oxidative stress or inflammation. 4-hydroxybenzoic acid is a phenolic antioxidant that scavenges free radicals; n-acetyl-tyrosine is an acetylated amino acid that may mitigate oxidative damage; Histamine is released during inflammation that synergizes with oxidative stress; and 4-hydroxyphenylpyruvic acid is a precursor to tyrosine metabolism which is linked to inflammation.

In sum, the enrichment of glycerophospholipid metabolism, together with other DEMs with oxidation-related properties observed in firefighters likely reflects the response to environmental stress-primarily oxidative stress-induced by fire-related toxicants. This metabolic shift is part of the cellular effort to repair damaged membranes and to regulate inflammatory responses. Understanding these changes not only sheds light on the acute biological responses to fire exposure but also highlights a potential pathway for understanding and improving long-term occupational health in female firefighters.

Among other interesting observations, nicotine was one of the DEMs that was reported significantly higher at postfire. Nicotine is the first biomarker for tobacco use. But given the short half-life of nicotine, cotinine has been used as the biomarker for exposure to cigarette smoke[117]. Nicotine was detected at both baseline and postfire in female firefighters and found increased after fire exposure (Figure 4.3), without the presence of cotinine, possibly indicative of other sources for nicotine than cigarette smoke.

4.4.2 Burn room/tower fires induced intense differential profiles in the post-exposure metabolome

In addition to the differences in burning materials, burn room/tower fire simulations typically involve dynamic tasks such as active searches and rescues, whereas flashover fire scenarios require firefighters to remain stationary. As expected, a broader differential metabolic profile was observed in firefighters exposed to burn room/tower fires compared to those exposed to flashover fires. However, without additional data, we cannot determine the relative contributions of burning materials and physical activity to the observed metabolic differences. Further research is needed to disentangle these factors and better understand their individual impacts on firefighters' metabolic responses.

4.4.3 Comparison with male firefighters

We previously reported metabolic differences before and after fire exposure in male firefighters who responded to municipal structure fires (MSFs) and identified a larger number of DEMs (N=268), and thus a broader metabolic response associated with fire exposure17. In one of our unpublished analyses of male firefighters responding to wildland-urban-interface (WUI) fires, we identified a broader set of metabolic responses to fire exposure, with 176 DEMs associated with fire exposure. The more pronounced response observed in male firefighters, compared to their female counterparts, is likely attributed to the nature of their fire exposure. Male firefighters in our other studies responded to actual fire incidents, which involved more intense and prolonged exposure, whereas female firefighters primarily participated in controlled training fires designed for simulation and instructional purposes. This difference in exposure intensity likely explains the weaker metabolic response profile observed in female firefighters.

Chapter 5. Conclusions

5.1 Summary of my work

This dissertation presents a series of urine-based metabolomics analysis to investigate the health effects of fire exposures among firefighters. The primary objective is to characterize the metabolic responses to fire exposure, which are essential for understanding both the acute and chronic health risks associated with this occupational hazard. Given that chronic health effects may not be observable in short-term studies, we interpret metabolic alterations in the context of established correlations from human studies to infer potential long-term implications.

Chapter 2 examines metabolic differences among male firefighters stratified by ethnicity. Our findings indicate significant metabolic variations, particularly enriched in galactose and tryptophan metabolism, between Hispanic and non-Hispanic firefighters. These differences may reflect chronic dietary or environmental exposures that contribute to disparities in cancer risk observed across ethnic groups within the firefighting community.

Chapter 3 explores the metabolic impact of wildland-urban interface (WUI) fires, focusing on the potential mechanisms underlying cancer and other health risks among WUI firefighters. We identified metabolic perturbations in urinary metabolites following WUI fire exposure and compared these findings to those associated with municipal structure fires (MSF). Our analysis reveals that WUI fires impose substantial physiological stress, disrupting a wide range of metabolic pathways and posing long-term health risks. While both WUI and MSF exposures induce similar metabolic disruptions, each fire type also elicits unique metabolic responses. The identification of altered metabolites provides valuable insights into the endogenous and exogenous chemical exposures encountered by firefighters. These findings highlight the need for further research into the long-term health implications of such exposures, particularly concerning cancer risk, and underscore the importance of targeted interventions to mitigate these occupational hazards.

Chapter 4 investigates the metabolic effects of training fire exposure among female firefighters. Our results indicate significant metabolic alterations, particularly in pathways related to oxidative stress and cellular damage. These findings suggest a potential link between repeated fire exposures and chronic inflammation, which may contribute to the increased prevalence of certain health conditions among female firefighters. Furthermore, our analysis suggests that the type of fires plays a critical role in shaping metabolic responses. Future research should aim to elucidate the connections between these metabolic alterations and disease development, as well as to further differentiate the health impacts associated with various fire types.

Together, these studies form a cohesive narrative that underscores the utility of metabolomics in capturing early biological responses to fire exposure, offering a window into the complex interplay between occupational hazards and firefighter health. By integrating diverse firefighter populations, fire types, and sex-specific analyses, this dissertation broadens the understanding of occupational exposure effects beyond conventional epidemiological measures. The recurring metabolic signatures, particularly those linked to oxidative stress, inflammation, and disrupted energy metabolism, emerge as consistent biomarkers of concern across studies. These converging findings not only validate the relevance of urine-based metabolomics in exposure assessment but also point toward shared biochemical pathways that may mediate long-term disease risk. Collectively, this work supports the development of exposure-specific surveillance tools and intervention strategies tailored to the unique demands of the firefighting profession.

5.2 Future directions

This dissertation highlights the complex metabolic responses to fire exposure in firefighters, providing insights into potential health risks associated with different fire types, gender, and ethnicity. However, several key areas warrant further investigation. Longitudinal studies are essential to establish direct links between acute metabolic perturbations and long-term health outcomes, such as cancer, cardiovascular disease, and respiratory conditions. Integrating multi-omics approaches, including proteomics, lipidomics, and transcriptomics, could provide a more comprehensive understanding of the biological pathways disrupted by fire exposure. Additionally, expanding research to include diverse firefighter populations, including volunteers, wildland firefighters, and those with varying exposure histories, would improve generalizability and help identify occupation-specific risks.

Future research should also focus on developing targeted intervention strategies to mitigate the adverse health effects of fire exposure. Identifying metabolic signals that serve as early biomarkers of exposure and disease risk could aid in the development of personalized monitoring and prevention programs. Moreover, studying the role of protective measures, such as improved personal protective equipment (PPE) and post-exposure recovery protocols, in mitigating metabolic disruptions could provide actionable strategies to reduce long-term health risks. By addressing these gaps, future studies can build on the foundation laid by this work to enhance firefighter health and safety.

5.3 Funding sources

The National Institutes of Health (NIH): National Institute of Environmental Health Sciences (NIEHS), National Cancer Institute (NCI); Federal Emergency Management Agency (FEMA), One Health Research Initiative (University of Arizona), United States Centers of Disease Control and Prevention (US CDC).

Appendix A. Urine metabolomics analytical workflow

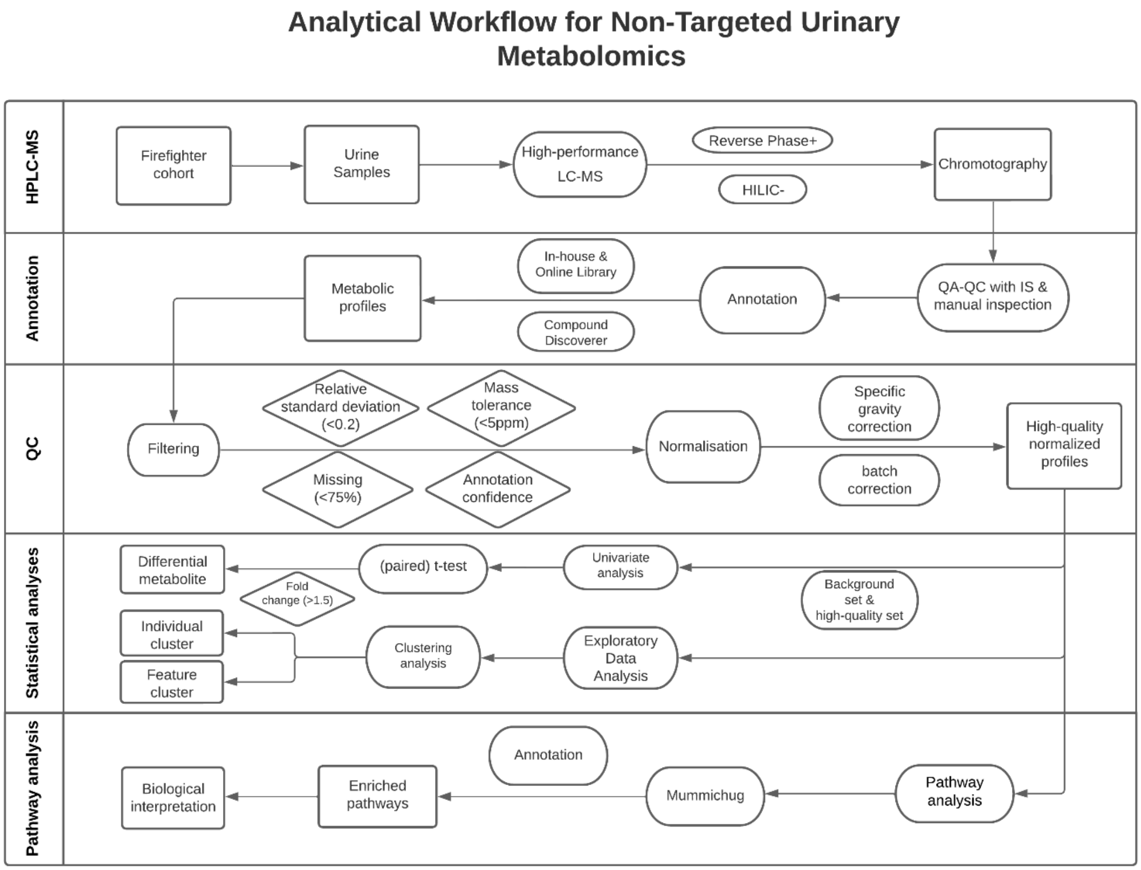


Figure A.1: Analytical diagram for urinary metabolomics analysis among firefighters. The analytical pipeline followed in this study was sketched in the Supplemental Fig. 5 where five components were involved: HPLC-MS, annotation, quality control (QC), statistical analyses, and pathway analysis. At the Analytical and Biological Mass Spectrometry Core, University of Arizona, HPLC-MS was input with pretreated urine sample from the FFCCS cohort and analyzed the samples in two separation-ESI modes: HILIC (-) and RP (+). The output MS files were then manually inspected against lab standards to ensure data quality and sent to Compound Discoverer for annotation against both in-house and online libraries afterwards. The pipeline incorporates a filtering process that incorporates relative standard deviation, mass difference, missing value percentage, and annotation confidence, to remove low-quality signals from further analyses. Specific gravity correction was applied to remove unwanted variation introduced by individual hydration level and batch correction was applied to further remove batch-wise variation. Remaining urinary features were input into differential analysis and exploratory analysis to identify differential hits and patterns that would help discern overall differential responses to the exposure variables among our samples. Pathway analysis took the complete set of urinary features as the reference set and the differential hits as the sample set to complete the overrepresentation analysis, and enrichment patterns were the output.

Appendix B. Supplementary figures

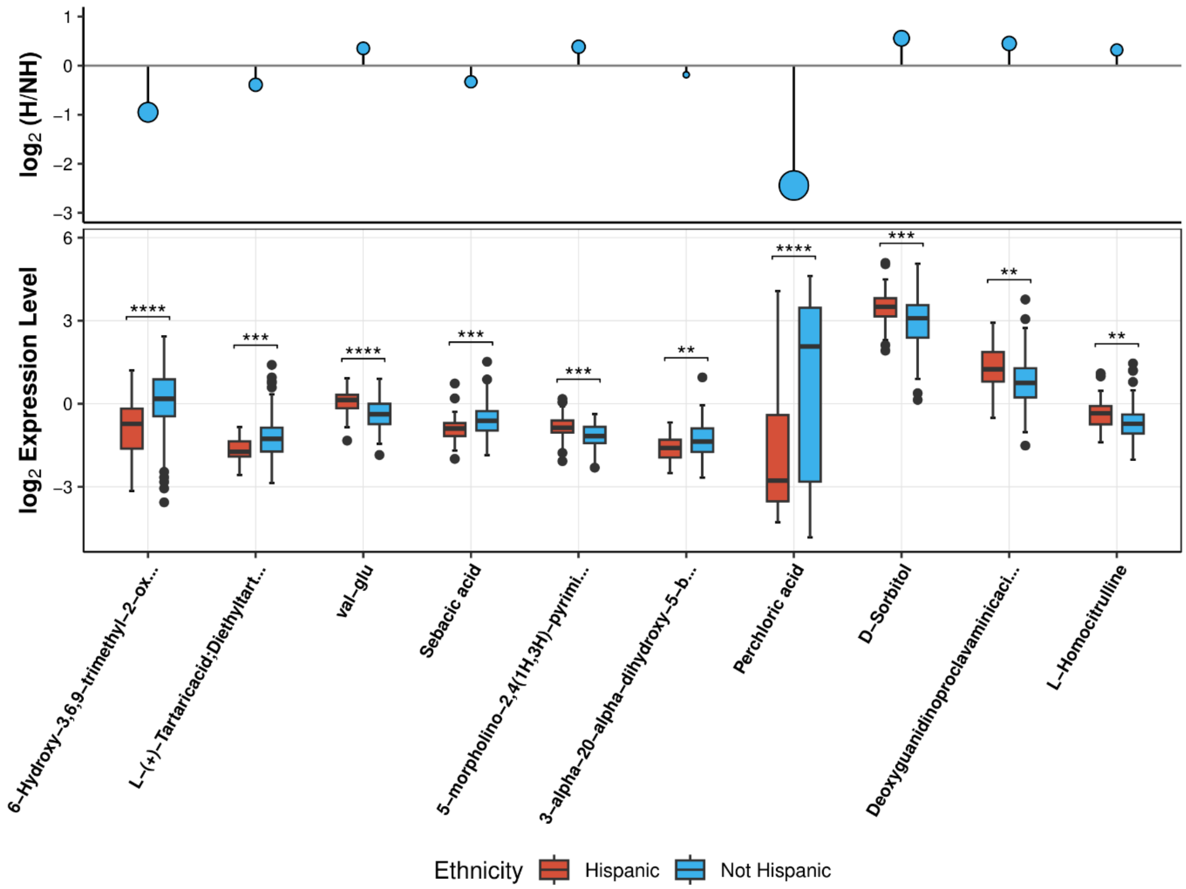


Figure B.1: Combo plot for model coefficients and expression level comparison for HILIC (-) differential metabolites with FDR < 0.05 and absolute slope ≥ 0.5. Top) Lollipop plot for model coefficients' size and direction comparing Hispanic samples to non-Hispanics where negative and positive y values indicated downregulation and upregulation, respectively; Bottom) Hispanic group was colored in red and non- Hispanic group was colored in blue. Asterisks indicated statistical significance as determined by the Wilcox test for two sample mean comparison; black dots indicated potential outliers.

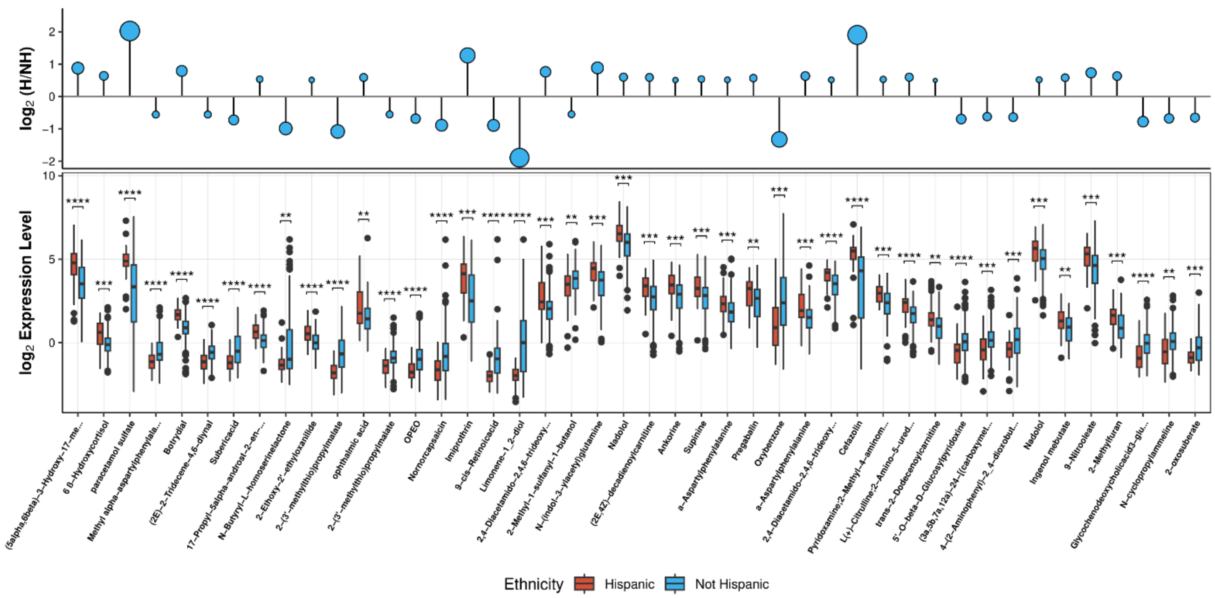
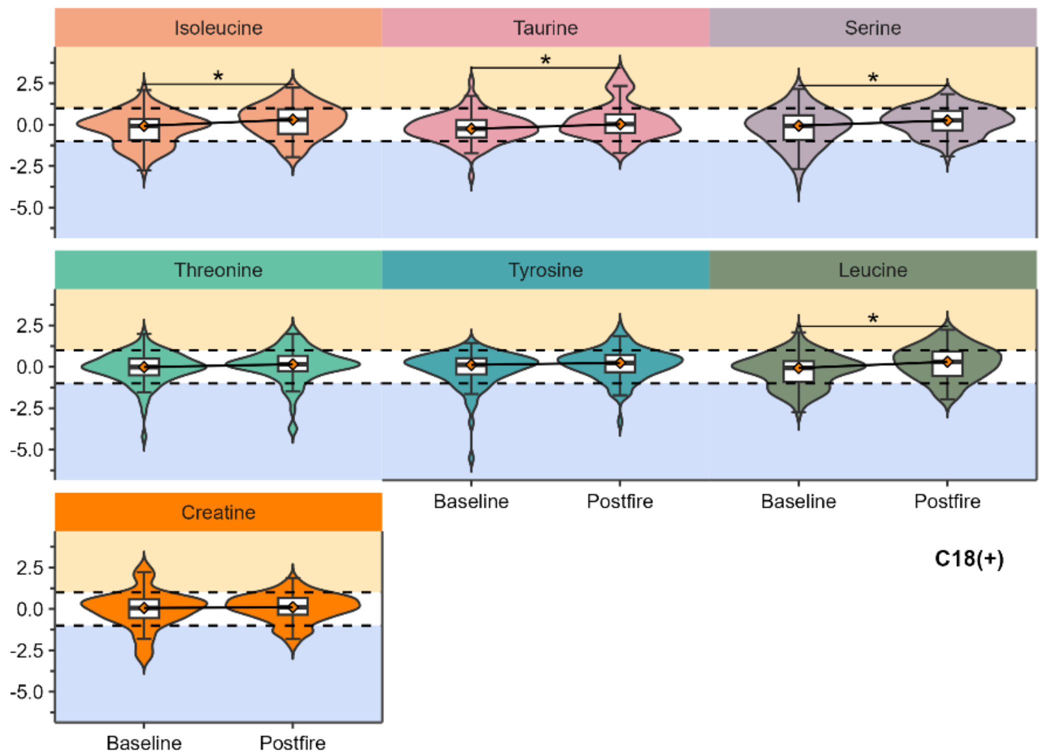
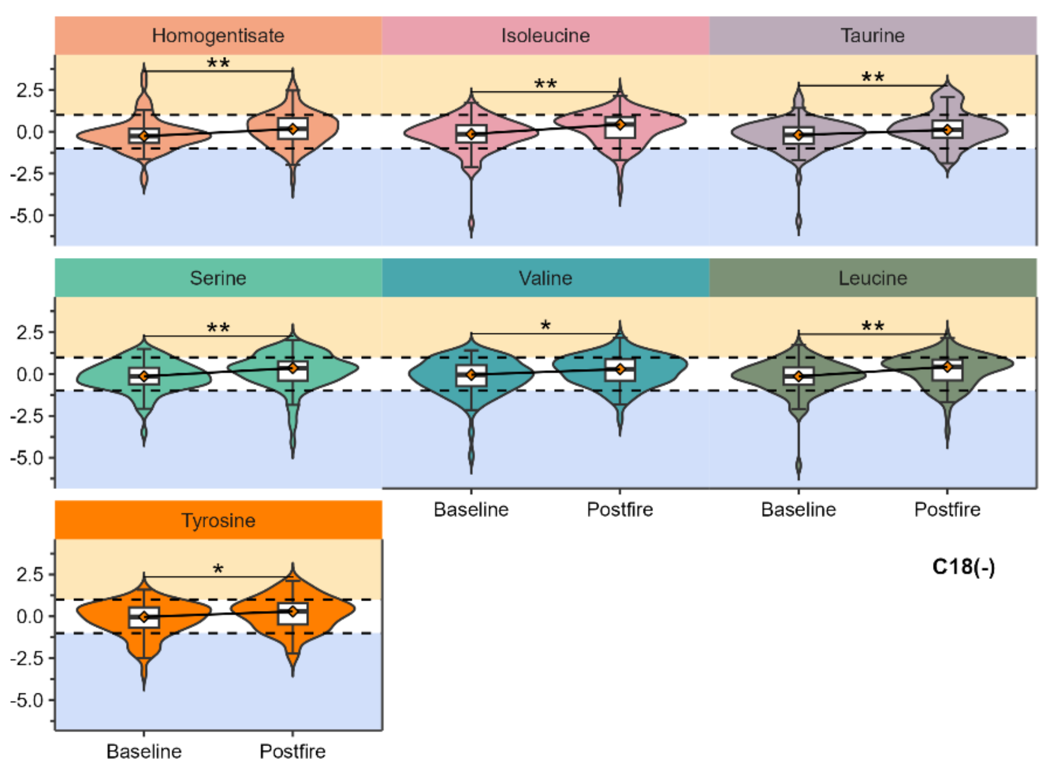


Figure B.2: Combo plot for model coefficients and expression level comparison for RP (+) differential metabolites with FDR < 0.05 and absolute slope ≥ 0.5. Top) Lollipop plot for model coefficients' size and direction comparing Hispanic samples to non-Hispanics where negative and positive y values indicated downregulation and upregulation, respectively; Bottom) Hispanic group was colored in red and non-Hispanic group was colored in blue. Asterisks indicated statistical significance as determined by the Wilcox test for two sample mean comparison; black dots indicated potential outliers.

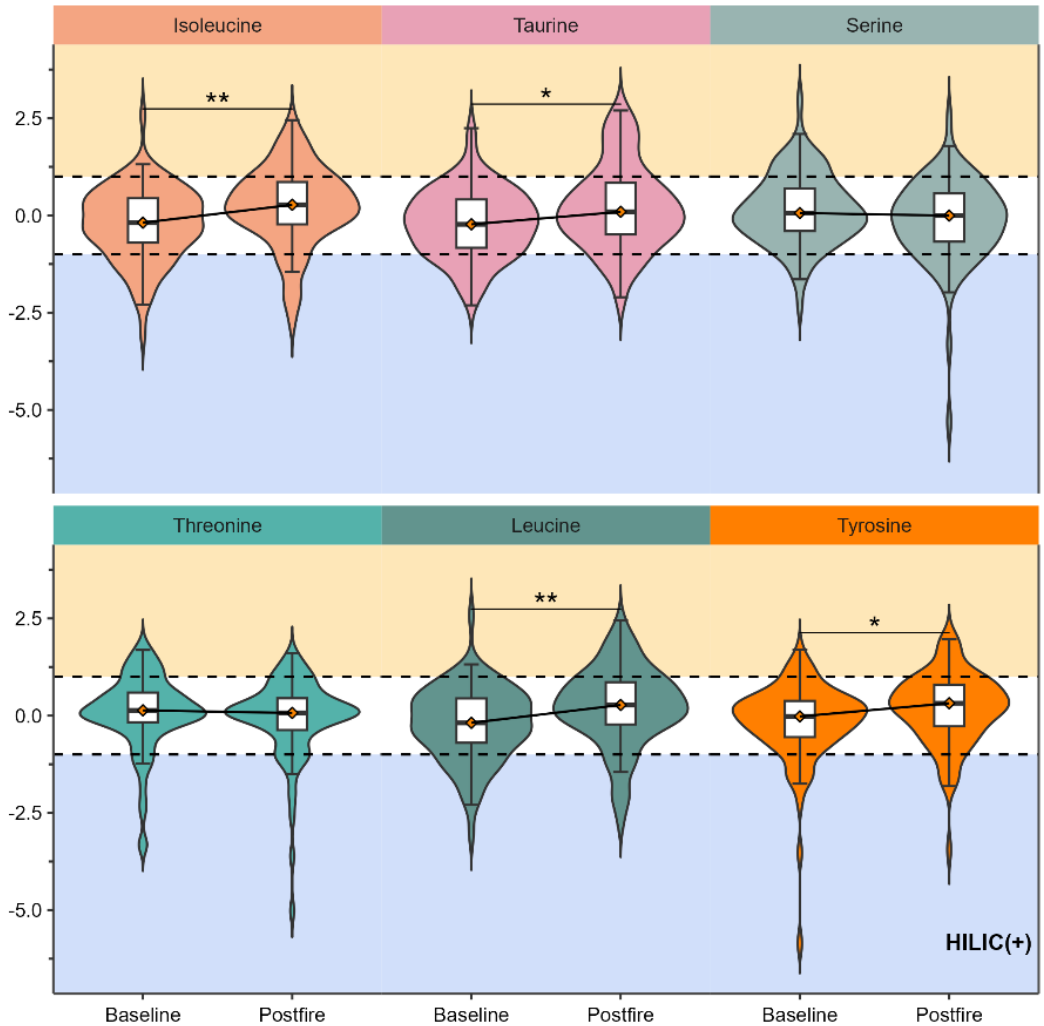


Isoleucine
Taurine
Serine
Threonine
Tyrosine
Leucine
Creatine
2.5
0.0
-2.5
-5.0
Baseline
Postfire
C18(+)



Homogentisate
Isoleucine
Taurine
Serine
Valine
Leucine
Tyrosine
2.5
0.0
-2.5
-5.0
Baseline
Postfire
C18(-)

Figure B.3: Combo plot for model coefficients and expression level comparison for RP (+) differential metabolites with FDR < 0.05 and absolute slope ≥ 0.5. Top) Lollipop plot for model coefficients' size and direction comparing Hispanic samples to non-Hispanics where negative and positive y values indicated downregulation and upregulation, respectively; Bottom) Hispanic group was colored in red and non-Hispanic group was colored in blue. Asterisks indicated statistical significance as determined by the Wilcox test for two sample mean comparison; black dots indicated potential outliers.


Isoleucine
Taurine
Serine
Threonine
Leucine
Tyrosine
2.5
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-2.5
-5.0
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HILIC(+)
Baseline
Postfire

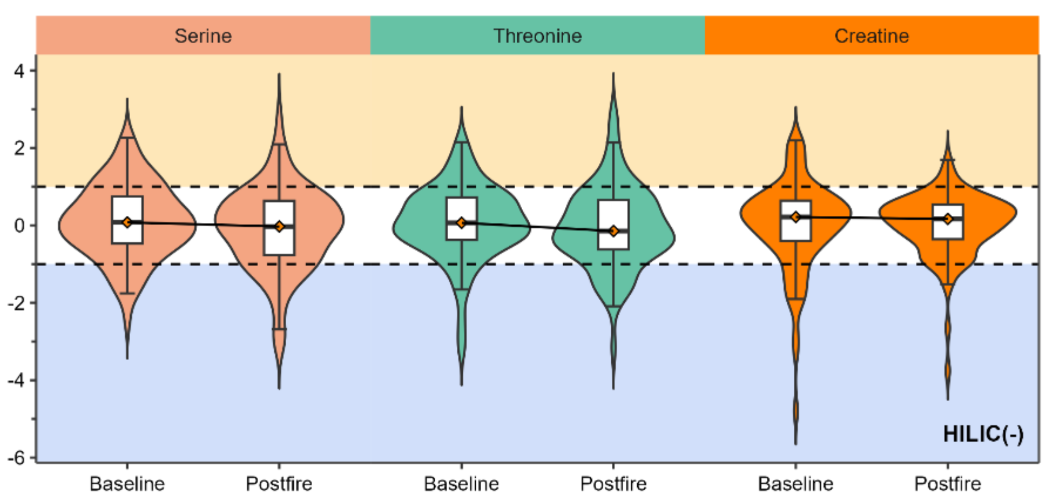
Serine
Threonine
Creatine
4
2
0
-2
-4
-6
HILIC(-)
Baseline
Postfire

Figure B.4: Combo plot for model coefficients and expression level comparison for RP (+) differential metabolites with FDR < 0.05 and absolute slope ≥ 0.5. Top) Lollipop plot for model coefficients' size and direction comparing Hispanic samples to non-Hispanics where negative and positive y values indicated downregulation and upregulation, respectively; Bottom) Hispanic group was colored in red and non-Hispanic group was colored in blue. Asterisks indicated statistical significance as determined by the Wilcox test for two sample mean comparison; black dots indicated potential outliers.

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