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THE EFFECTS OF PER- AND POLYFLUOROALKYL SUBSTANCES (PFAS) AND HIGH FAT DIET ON METABOLISM, REPRODUCTIVE HEALTH & PROSTATE CANCER PROGRESSION

BY

OZAN BERK IMIR

THESIS

Submitted in partial fulfillment of the requirements for the degree of Master of Science in Nutritional Sciences in the Graduate College of the University of Illinois at Urbana-Champaign, 2021

Urbana, Illinois

Master’s Committee:

Professor Jodi A. Flaws, Chair
Associate Professor Zeynep Madak-Erdogan, Director of Research
Professor Joseph M. K. Irudayaraj
ABSTRACT

Per- and polyfluoroalkyl substances (PFASs) are a group of synthetic chemicals that have been utilized in various industries and settings. Their resistance to water, oil, grease, and extreme temperatures enabled utilization of PFAS in adhesives, cleaning-products, paints, coatings, packaging, building materials, and firefighting foams. Due to highly fluorinated carbon chains ranging in length, PFAS are resistant to decomposition, and have increased longevity in media, such as soil, water, dust, and air. Because of increased stability in the environment, many communities in the US are exposed to high concentration of PFAS. Multiple studies reported increased PFAS blood concentrations in fluorochemical factory workers, office workers, and firefighters, all of which are exposed to these materials on a day-to-day basis. Epidemiological studies linked PFAS exposures to increased risk of various pathological conditions including non-alcoholic fatty liver disease, infertility, and cancer. Epidemiological studies suggested a link between increased blood PFAS levels and prostate cancer incidence, but the mechanism of action for such a phenomenon is unknown. In the present thesis research, we investigated how PFAS impacts prostate cancer metabolism, progression, and prognosis. We tested the hypothesis that metabolic alterations from high fat diets combined with PFAS exposures play a significant role in prostate cancer initiation and tumor progression.
ACKNOWLEDGEMENTS

I would like to offer my sincere appreciation to my mentor Dr. Zeynep Madak-Erdogan for giving me all the time, effort, and dedication she has put into the culmination of my project and my education. I am grateful for the guidance that she has given, and the helping hand she has offered many times when I needed it most. I would also like to thank Dr. Jodi Flaws and Dr. Joseph Irudayaraj for being in my committee, helping me develop this project and facilitating the creation of this document.

To all current and former M-Lab members: Eylem Külköylüoğlu-Çotul, Qianying Zuo, Ashlie Santaliz-Casiano, Alicia Arredondo-Eve, Justina Zurauskiene, Ayca Nazli Mogol, Brandi Smith, Shoham Band, Kinga Wrobel, Yiru Chen Zhao, Karen Chen, and Kadriye Hieronymi, thank you so much for your support on this project and your friendship. I will dearly miss all the great conversations and cherished moments that we have had together. It was a pleasure and a privilege to work with you all. I would also like to thank our undergraduate students Alanna Zoe Kaminsky, Sruthi Sridhar, Haighth Colette Connors, Matthew Chanho Jin, Nina Litvak, and Saumya Agrawal. Despite the COVID-19 pandemic, you have exceeded all expectations for which I am proud to have worked with you, and with the determination you have at hand and the help of M-Lab, I have no doubt that you will not only achieve but also outperform your goals in life!
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CHAPTER 1
INTRODUCTION

Per- and polyfluoroalkyl substances (PFAS) are fluorocarbons with a carbon backbone flanked with fluorine atoms and capped with a carboxyl group. PFASs adhere to metal, plastic, or other designated charged surfaces by virtue of their more electronegative carboxylic acid group and polymerize with other PFAS compounds via the fluorine atoms on their long carbon chains, forming a stain-repellant surface coat which renders the surface inaccessible (Behr, Plinsch, Braeuning, & Buhrke, 2020; Domingo & Nadal, 2019; Ojo, Xia, Peng, & Ng, 2021; Sunderland et al., 2019). PFAS compounds are frequently used in many industries as stain-repellant coating material on food packaging and grime-free cooking equipment such as Teflon pans as well as being coated into pipes to make them leak-proof (Sunderland et al., 2019).

Perfluoroalkyl substances, both within their manufacture and in their application onto coating surfaces, pose a threat against human health. Soil, water, and air contamination; direct contact to coated surfaces; and the consumption of food that has had contaminant exposure are some of the ways by which one can get exposed to PFAS compounds (Legoff et al., 2021; Mahinroosta & Senevirathna, 2020; Temkin, Hocevar, Andrews, Naidenko, & Kamendulis, 2020). Due to their low manufacturing cost and wide range of use-cases, PFASs come in many forms. Perfluorooctane sulfonate (PFOS), perfluorobutane sulfonic acid (PFBS) and perfluorooctanoic acid (PFOA) are among the most readily used and the most environmentally persistent of these pollutants. Due to the health risks involved with the use of PFASs, many environmental and public health institutions such as the EPA and NIH have issued prompt advisories following the emergence of the scientific data (Agency, 2021; Sciences, 2021). Many industries have since been trying to develop safer PFAS alternatives that could replace these
flagged chemicals. Hence, other frequently used types of PFAS compounds such as perfluorononanoic acid (PFNA), perfluorohexane sulfonic acid (PFHxS) and perfluorodecanoic acid (PFDeA) were developed to serve this purpose, but all of them have also been detected as persistent environmental contaminants found mostly in drinking water and surface soil (Mahinroosta & Senevirathna, 2020).

PFAS exposure was found to cause various cellular and systemic metabolic alterations. A 2016 study by researchers from Nanjing University in China revealed that the liver metabolism of mice that were exposed to PFAS was altered such that the amino acid and TCA cycle dependent energy metabolism were statistically significantly shifted (Costello & Franklin, 2006; Yu et al., 2016). In addition, a 2012 study published in Environmental Science & Technology used a proteomic label quantification technique called iTRAQ to visualize biomarkers of toxicity when mice were exposed to PFOS, which led to the discovery of both novel toxicity biomarkers and revealed that PFOS exposure significantly upregulated peroxisomal $\beta$-oxidation controlling enzymes (F. Tan et al., 2012). With increased dose of PFOS concentrations (5.0 mg/kg BW), higher peroxisome (23.5-fold, $p$-val<0.05) endoplasmic reticulum (10.1-fold, $p$-val<0.05), mitochondria (3.9-fold, $p$-val<0.05) and membrane protein (1.6-fold, $p$-val<0.05) concentrations were observed (Domazet, Grøntved, Timmermann, Nielsen, & Jensen, 2016). Furthermore, PFAS exposure was found to dysregulate multiple lipid metabolism pathways (glycosphingolipid metabolism, fatty acid metabolism, de novo lipogenesis, and linoleic acid metabolism), as well as amino acid metabolic pathways (aspartate and asparagine, tyrosine, and arginine and proline metabolism) (Alderete et al., 2019; Qu et al., 2020). PFAS can, therefore, impact lipid metabolism and alter cellular energetics which may have detrimental health outcomes.
In addition to the cellular shifts observed in in vitro and in vivo settings, PFAS was shown to trigger systemic changes in patient metabolisms as well. GWAS studies in exposed patients have historically shown a positive correlation between PFAS and metabolites such as fatty acid and glycerophospholipids (Thomas et al., 2008). Additionally, a Metabolomics journal publication from 2019 featuring a HILIC-coupled LC/MS metabolomics analysis of 114 children showed that children with low to moderate PFOA, PFOS, PFNA and PFHxS concentrations in their serum exhibited altered arginine, proline, aspartate, asparagine, butanoate, glycine, serine, alanine, and threonine metabolism (Kingsley et al., 2019).

Lifestyle factors and environmental exposures impact prostate cancer risk. Although chronic exposures to PFAS are associated with elevated the prostate cancer incidence and/or mortality, the cellular targets and potential mechanisms are unknown. Using a combination of human prostate and relevant human prostate cancer models, spatial and molecular data, and sophisticated analytical tools, my thesis provided some key mechanistic insights into prostate carcinogenesis as a function of PFAS plus high fat diet exposures: metabolic changes in benign or tumor cells that lead to epigenomic reprogramming and altered signaling, which ultimately increase tumorigenic risk and tumor aggressiveness.

In my thesis project, the effects of PFAS on prostate cancer proliferation, growth progression and metabolic activity were explored. To understand how the metabolic landscape is shifted upon PFAS exposure, a metabolomics approach was employed. An animal study focused on the growth rate of tumors in animals that were exposed to PFOS, a type of PFAS compound, and high fat diet that may affect liver health in combination with PFAS. This research will help us gain preliminary knowledge on the potential dangers of PFAS exposure and enhance our understanding on how it may impact reproductive cancer characteristics.
The overall *objective* in the present thesis project was to characterize the actions of PFAS and its interaction with high fat diets that together drive prostate carcinogenesis (Figure 1). As such, we hypothesized that PFAS exposures together with high fat diets drive metabolic changes that reprogram prostate cells through epigenetic modifications that, independently or combined, initiate prostate cancer and promote tumor progression. Our studies unraveled *new and clinically relevant insights regarding* novel metabolic and epigenetic states that provide a rational framework for future development of effective preventative, and therapeutic strategies for PFAS induced prostate cancers.
CHAPTER 2

PERFLUOROALKYL AND POLYFLUOROALKYL SUBSTANCES AND EFFECTS OF EXPOSURE ON METABOLIC AND REPRODUCTIVE HEALTH

2.1. LINK BETWEEN PFAS EXPOSURE AND PROSTATE CANCER RISK

2.1.1. Per- and polyfluoroalkyl substances:

PFAS are a family of chemicals containing long hydrophobic (8-carbon) chains fully saturated with fluorine atoms (i.e., perfluoroalkyl chains) and a hydrophilic polar functional group (Prevedouros, Cousins, Buck, & Korzeniowski, 2006). These chemicals are thermally stable and lipid- and water-repelling, prompting their extensive use since the 1970s in household products such as carpets, Gortex products, and non-stick cookware. Such widespread usage has led to accumulation of these persistent organic pollutants in the environment (Fraser et al., 2013; Karásková et al., 2016; Oakes et al., 2010; Rappazzo, Coffman, & Hines, 2017). Two PFAS, perfluorooctanoate acid (PFOA) and perfluorooctanesulfonic acid (PFOS), are the most abundant in the environment and are a major source of exposure to humans through food and drinking water. Unfortunately, these and newer PFAS substitutes (e.g., GenEx) do not readily degrade and are commonly referred to as “forever chemicals”. An estimated 99% of Americans are exposed to and/or have these chemicals in their body (Calafat et al., 2019; Kato, Wong, Jia, Kuklenyik, & Calafat, 2011). Wastewater treatment plants are a major sink for PFAS by receiving PFAS-impacted wastewater. Landfill leachate, wastewater effluent, biosolids application, and groundwater recharge using surface runoff re-introduces PFAS to water sources including drinking water. Crucially, PFAS exposures exert negative effects on health.
2.1.2. Prostate cancer and increased risks from environmental factors

Prostate cancer is the most commonly diagnosed noncutaneous cancer in American men and the second leading cause of cancer-related deaths (Siegel, Miller, & Jemal, 2020), although its etiology remains elusive. Our collaborators at UIC, previously determined that exposure to two well-established endocrine disruptors/environmental toxicants, bisphenol A (BPA) and arsenic, increases prostate cancer risk and identified molecular mechanisms that underpin these activities, including prostate stem-progenitor cell (SPC) reprogramming through epigenetic modifications (Ho et al., 2015; Xie et al., 2020). In addition to environmental and occupational exposures, lifestyle factors including diet and body weight dictate overall increased prostate cancer risk (Freedland & Aronson, 2004; Narita et al., 2019). Relevant to this thesis, epidemiology studies show that prostate cancer risk and mortality increase with PFAS exposure (Barry, Winquist, & Steenland, 2013; Kirsten T. Eriksen et al., 2009; Gilliland & Mandel, 1993) as well as with obesity (Hardell et al., 2014; Vidal et al., 2020). Despite this evidence, mechanistic data on the molecular underpinnings of PFAS chemicals in the prostate are limited/nonexistent. It is well-established that metabolic adaptations in prostate cancer alter the epigenetic landscape, in part due to changes in substrate availability for epigenetic enzymes (detailed below). Further, epigenetic marks dictate the activity of PPARα, a critical transcription factor in PFAS-associated carcinogenesis (Stanifer et al., 2018; Wolf et al., 2008) that is liganded by both PFAS (Evans, 2020.) and metabolites associated with HFDs (David Patsouris, Reddy, Müller, & Kersten, 2006).
2.2 MOLECULAR MECHANISMS OF METABOLIC DEREGULATION BY PFAS EXPOSURE

PFAS are not thought to be genotoxic (Eriksen et al., 2010; Freire et al., 2008; Lindeman et al., 2012), suggesting that PFAS elicit effects without causing direct DNA mutations. One plausible means through which PFAS may exert effects is epigenetic and transcriptomic alterations. Associations between PFAS exposures and altered methylation, either genome-wide or at specific histone loci, are described from other laboratories and our collaborators (Rashid, Ramakrishnan, Fields, & Irudayaraj, 2020; Tian et al., 2012; Wan et al., 2010; Wen, Mirji, & Irudayaraj, 2020). PFAS exposure is also associated with lower global DNA methylation in neonates (Guerrero-Preston et al., 2010; Kobayashi et al., 2017; Watkins et al., 2014).

2.2.1 Link between systemic metabolism and hormone-dependent cancers

The metabolic status of cancer cells determines phenotypic characteristics and drug responses of hormone-dependent cancers (Cotul et al., 2020; Kulkoyluoglu-Cotul et al., 2019; Madak-Erdogan et al., 2019; Madak-Erdogan et al., 2013). Of note, a recent study showed that high fat diets (HFDs) induce changes in one-carbon metabolism, impact histone methylation marks, and increase prostate cancer progression in an in vivo model of MYC-induced prostate cancer (Labbé et al., 2019). Previously, using a unique multi-omics approach and serum samples from obese and non-obese individuals, our laboratory identified obesity-associated factors that increase breast carcinogenesis (Madak-Erdogan et al., 2019). We showed that free fatty acids work through PPARα to rewire breast cancer metabolism and affect ERα cistromes that alter transcriptomes. Structurally, PFAS resemble free fatty acids and bind to the same sites on serum proteins. Several studies found that PFAS chemicals work through PPARα to impact metabolism and the immune system (DeWitt et al., 2009; Stanifer et al., 2018; Wolf et al., 2008).
Studies have shown that multiple types of perfluoroalkyl substances have activated variants of peroxisome proliferator-activated receptors. Researchers from the German Federal Institute for Risk Assessment investigated the impact of various PFASs on the activation of PPARα through their dependent target genes, as well as its effect on eight other human nuclear receptors by using luciferase-based reporter gene assays (Kunath et al., 2015). The study observed activation of PPARα when exposed to PFOA, PFOS, PMOH (“Gen X”), PFHxS, PFBA, PMPP, and PFHxA, but PFOA and PMOH exhibited the strongest impact of activation. PPARγ was the only other human nuclear receptor found to be activated by PFASs (PMOH and PMPP) of those that were tested. This study provides additional research on the impact of PFASs on PPAR pathways (Behr et al., 2020). Another study was done by researchers from the Chinese Academy of Science that examined the structural impact of 16 PFAS binding affinity and activation potency on PPARγ using fluorescence polarization based competitive binding assays and Hep G2 cells. The experiments showed a high expression and activation of PPARγ correlated with medium-sized PFC chain lengths (PFOA, PFDA). This study demonstrated another case of PFC activation of PPARγ (Zhang, Ren, Wan, & Guo, 2014). Researchers from the University of Minnesota Medical School sought to determine the activation of PPARα in primary rat and human liver cell cultures by exposing them to 25μM of PFAS for 24 hours. They found relative activation potencies by many PFASs with the carboxylic acids induced a greater response compared to sulfonates as follows: perfluorononanoic acid ≥ PFOA > PFDA > perfluorohexanoic acid > PFBA and perfluorohexane sulfonate > PFOS > PFBS. Activation of mouse PPARα showed to be statistically greater than the human PPARα (Bjork & Wallace, 2009). In conclusion, these studies contribute evidence towards the influence of PFOS and PFOA on the activation of PPARα.
PFOA and PFOS have been found to activate human and mouse peroxisome proliferator-activated receptor alpha. A study done by researchers from the United States Environmental Protection Agency determined the ability of PFOS and PFOA to activate PPARs using transient transfection cell assays. COS-1 cells that were cultured with mouse or human PPARα, β/δ, and γ reporter plasmids and exposed to PFOS and PFOA for 24 hours displayed an increase in PFOA transactivity compared to PFOS in both human and mouse receptors (Takacs & Abbott, 2007). In addition to these findings, the study reported that PFOA activated both human (p < 0.05; 30 and 40μM) and mouse (p < 0.05; 10, 20, 30, and 40 μM) PPARα in a dose-dependent manner, whereas PFOS activation of PPARα only occurred in the mouse construct (p < 0.01; 120μM). PPAR β/δ was activated by PFOA and PFOS in the mouse construct only (p < 0.05; p < 0.05) for both PFASs. PFOA and PFOS did not activate the mouse or human PPARγ (Takacs & Abbott, 2007). A study by researchers from Boston University focused on determining the impact of activation of PFOSAs containing PFOS on mouse and human PPARα using COS-1 cells cell-based luciferase reporter trans-activation assays. PFOS activated both mouse and human PPARα in a dose-dependent fashion (p < 0.05), with a maximum activation from 4-6-fold. PFOS was also tested on FAO, a rat hepatoma cell line, which showed a dose-dependent induction of PTL protein expression (p < 0.05), a common PPARα-dependent response (Shipley et al., 2004). Another study conducted by researchers from the United States Environmental Protection Agency examined the effects of PFOA on PPARα, β, and γ expression and PPAR-regulated genes by orally dosing pregnant CD-1 mice. PPARα mRNA was found to be expressed at higher levels than PPARβ or PPARγ (p < .001) when exposed to PFOA. PPARα was dominant with PFOS exposure in the following types of tissues: liver, heart, kidney, thymus, adrenal, and intestines (Abbott et al., 2012). These studies provide statistically significant data that support
the activation of PPARα by PFOS and PFOA. Examining the specific types of PFAS activation on PPAR can provide essential insight on what to avoid for the usage of PFAS in firefighting foams. Overall, these studies support and suggest that PFAS exposure increase the risk of prostate cancer via activation of PPARs.
CHAPTER 3

PFAS EXPOSURE LEADS TO INCREASED RATE OF TUMOR PROGRESSION

3.1. INTRODUCTION

It is well-documented that PFASs, mainly PFOA and PFOS, may act as an endocrine disruptors, and lead to abnormalities in pathways regulating steroidogenesis, cholesterol homeostasis, pregnancy, weight maintenance, and molecular toxic waste disposal (Blake et al., 2020; Dennis et al., 2020; Poteser, Hutter, Moshammer, & Weitensfelder, 2020). The effects of PFAS on reproductive cancers, however, was the main focus of this study.

To discern any possible correlation between PFAS and cancer, it is essential to outline the molecular and clinical characteristics of male reproductive cancers. Male reproductive cancers may present themselves as either testicular cancers or prostate cancers, and prostate cancer is the most common type of male cancer in the United States, with 248,530 new cases and 34,130 deaths in 2021 so far, amounting to a prevalence of 1 in every 8 men having the risk of being diagnosed with prostate cancer throughout their lives (team, 2021). Contrary to testicular cancer, which presents itself frequently between 20 to 35 years of age, prostate cancer risk tends to increase in the later stages of life, with around 60% of the cases being older than 65 (Grossman et al., 2018). This is likely due to the dysregulation of testosterone production with increasing age. Interestingly, PFAS exposure tends to have a similar effect on physiological testosterone metabolism (Bandak et al., 2018; Gann, Hennekens, Ma, Longcope, & Stampfer, 1996; Kokontis et al., 2005; Miura et al., 2020; Watts et al., 2018). Male mouse pups exposed to PFAS in utero can have an approximately 50% reduction in their serum testosterone levels, and genes associated with apoptosis and cell-to-cell junctions may get either inhibited or
transcriptionally dysregulated, which can create a tumorigenic microenvironment that may facilitate the development of reproductive cancers (Zhong et al., 2016).

As PFAS exposure was found to be associated with liver metabolism dysfunction and development of NAFLD, the association between a high fat diet consumption that can create an imbalance in cholesterol metabolism (Lysne et al., 2016; X. Tan et al., 2013; Venkateswaran & Klotz, 2010) was introduced in combination with PFAS to an immunocompromised mouse model to test the detrimental effects of PFAS-high fat diet combination on prostate cancer progression. To facilitate human prostate tumor survival in this mouse model, we implanted a testosterone tube dorsomedially to increase the serum testosterone levels of mice. With testosterone added to the in vivo model, we used dihydroxytestosterone (DHT) as a control in all subsequent in vitro studies. In preliminary negative tests, the proliferative characteristic that PFAS seemed to promote in prostate cancer cells was not observed in the absence of DHT, so DHT control proved to be an essential component to the prostate cancer-PFAS paradigm. We hypothesized that our PFAS treated subjects, would have accelerated tumor progression and coincident in vitro models with added DHT would have a more proliferative prostate cancer profile upon PFAS introduction due to a shift in PPARα-mediated epigenetic and metabolic alterations.

3.2. MATERIAL AND METHODS

Cell Culture

RWPE1, RWPE-Kras (RWPE2) cells were a gift from Dr. Gail Prins from University of Illinois, Chicago. Cells were maintained in Gibco Keratinocyte SFM 1X growth media with glutamine (Gibco 17005042, Fisher Scientific, Waltham, MA 02451).

WST-1 In vitro Cell Viability Assay
RWPE-kRAS and RWPE1 prostate cancer cells were maintained as described above. The day before the treatments, cells were seeded at a density of 5000 cells/well in a 96-well plate. Next day, cells were treated with varying concentrations (10^{-5} \text{ M}, 10^{-6} \text{ M}, 10^{-7} \text{ M}, 10^{-8} \text{ M}, 10^{-9} \text{ M}, 10^{-10} \text{ M}) of PFOS or PFBS with or without 1 nM dihydrotestosterone (DHT) (2 biological replicates, 6 technical replicates). Treatments were repeated again after two days. Effects of PFAS on cell viability was quantitated by using the WST-1 reagent treatment, followed by an incubation period of 45 minutes. Absorbance readings were measured at 450nm using Cytation5 plate reader (BioTek, Winooski, VT, USA), and statistical analyses were performed using Graphpad® Prism8 software (GraphPad Software Inc., La Jolla, CA, USA, www.graphpad.com).

**In vivo Prostate Cancer Xenograft Models**

Animal experiments and protocols were approved by the University of Illinois at Urbana-Champaign and the National Institutes of Health standards for the use and care of animals (IACUC Protocol #20159) were followed. RWPE-1 and RWPE-KR as prostate cancer epithelial cell lines were used for the tumor xenograft study. 4-week-old athymic nude male mice (RRID:RGD_5508395) were ordered from Jackson laboratory (stock no. 007850). After a week of acclimatization, to test synergy between PFAS and HFD, we compared carcinogenesis in mice fed a high fat diet vs control diet. Because standard diets contain isoflavones with estrogenic activity that interferes with metabolic effects, we used purified (AIN93M) diets. Purified HFDs for our studies (Harlan TD.88137), aka the “western diet”, are high in butterfat (~42% Kcal from fat) and sucrose, with a modest level of cholesterol (0.2%). This diet is commonly used for metabolic syndrome studies.

After 10 days, 2 x 10^6 RWPE-Kras cells were injected to the left and right flanks of the mice. In addition, testosterone sialistic tubes were implanted to provide continuous testosterone,
which is needed for xenograft establishment and growth. Animals were administered PFOS by oral gavage seven days per week at 10 µg/kg/mice. Food consumption and animal weights were monitored twice weekly. Tumor size measurements were performed three times a week using a digital caliper. Animals were sacrificed five weeks after the initial cancer cell line injection. Tumors, livers, prostates, and blood were harvested and were flash frozen or were fixed in formalin for future staining.

**OMICS-Based Metabolic Profiling**

RWPE-Kras cells were seeded in growth media. The next day, they were treated with Veh (cell growth media), 5mL of 10^{-8} M PFOS, 5mL of 10^{-8} M PFBS with or without 10^{-9} M DHT. Cellular metabolites were extracted using a 1:2:1 mixture of Acetonitrile:Isopropanol:Water. Extracts were sent to University of Illinois at Urbana-Champaign’s Metabolomics Core Facility to detect and quantify metabolites using Gas chromatography mass spectroscopy (GC/MS) analysis. Metabolic profiles were obtained from Agilent GC-MS system (Agilent 7890 gas chromatograph, an Agilent 5975 MSD, and an HP 7683B autosampler, Lexington, MA, USA). The spectra of all chromatogram peaks were evaluated using the AMDIS 2.71 and a custom-built database with 460 unique metabolites. All known artificial peaks were identified and removed prior to data mining. Individual metabolomic data sets for each treatment were separated and grouped into files to make comparisons between treatment conditions using web based Metaboanalyst software. The sample class annotations consisted of Veh vs. PFOS, Veh vs. PFBS, Veh vs. DHT, DHT vs. DHT + PFOS, and DHT vs. DHT + PFBS. These files were uploaded to the Enrichment Analysis tool of MetaboAnalyst software version 5.0 (RRID:SCR_015539). The data was not normalized, transformed, or scaled, but it was compared to the SMPDB reference metabolome which represents metabolite values.
from normal metabolic human pathways. The top 25 enriched metabolic pathways and associated metabolites were retrieved along with their p-values and enrichment ratios. Heatmaps were developed for each treatment group based on class averages using the default settings for clustering, and the data was restricted to the top 25 metabolites using PLS-DA VIP.

For sequencing-based transcriptome/RNA analysis, RWPE-kRas cells were treated with Veh or 10 nM PFAS with and without 1 nM DHT for 24 hours. cDNA libraries were be prepared and sequencing reactions evaluated at the UIUC Sequencing core. Processing of data and analysis was be performed as previously described (Madak-Erdogan et al., 2019; Madak-Erdogan et al., 2013; Madak-Erdogan et al., 2016; Madak-Erdogan, Lupien, Stossi, Brown, & Katzenellenbogen, 2011; Madak-Erdogan, Ventrella, Petry, & Katzenellenbogen, 2014; Zhao & Madak Erdogan, 2016). GSEA analysis was performed to identified enriched gene set grouping as previously described (Madak-Erdogan et al., 2013; Madak-Erdogan et al., 2016; Zhao & Madak Erdogan, 2016).

**Plate-Based Pyruvate and Acetyl CoA Assays**

We prepared metabolite extracts from aforementioned *in vitro* cell models and xenograft tumors to *validate* changes in pyruvate and acetyl-CoA levels using fluorescence based plate assays (Sigma, #MAK071 and #MAK039). RWPE-Kras cells were seeded in 10 cm plates at a density of 500,000 cells/plate and were treated with Veh (cell growth media), 5mL of $10^{-8}$ M PFOS or 5mL of $10^{-8}$ M PFBS with or without $10^{-9}$ M DHT for 24 hours. The pyruvate concentrations in the cells were detected by Cytation 5 plate reader upon formation of fluorescent metabolite as pyruvate underwent oxidation by pyruvate oxidase. Pyruvate concentrations were in nmol/µL.
For Acetyl CoA measurement, the fluorescence produced from NADH and probe reaction that is coupled to conversion of Acetyl-CoA to CoA was detected using Cytation 5 plate reader. Each experiment was repeated twice, with three technical replicates.

**Western Blotting for Epigenetic Markers’ Assessment**

RWPE-Kras cells were seeded at a density of 500,000 cells/plate on 10 cm plates. Cells were treated with Veh (cell growth media), 5mL of $10^{-8}$ M PFOS or 5mL of $10^{-8}$ M PFBS with or without $10^{-9}$ M DHT for 24 hours. Cell lysates were collected in lysis buffer (0.5 M EDTA, 1 M TrisHCl pH 8.1, 10% SDS, 10% Empigen, ddH2O) with 1X Complete Protease Inhibitor (Roche) and 1X Phosphatase Inhibitor (Thermo Scientific, Waltham, MA, USA). Cell lysates were sonicated and protein concentrations were determined by BCA assay (Thermo Scientific). Samples were boiled in SDS-containing loading buffer and each sample was run in 10% precast gels (BioRad) and transferred to nitrocellulose membrane. The membranes were blocked in Blocking Buffer (Odyssey®, Li-Cor, Lincoln, NE, USA) and target proteins were probed with Acetyl Histone Antibody Sampler Kit (#9933, Cell Signaling) (RRID:AB_10699455), Tri-Methyl Histone Antibody Sampler Kit (#9783, Cell Signaling) antibodies in 1:1000 dilution and $\beta$-actin (Sigma SAB1305546) (RRID:AB_2541177) antibody in 1:10000 dilution. The secondary antibodies obtained from Odyssey were used at 1:10000 dilution. The membranes were visualized by using Licor Odyssey CLx infrared imaging device and software.

**3.3. RESULTS**

**3.3.1. Exposure to PFAS increases rates of cell proliferation in malignant prostate cancer cell lines in vitro.**

Epidemiology studies suggest an increase in prostate cancer incidence and/or mortality with increasing years of chronic occupational PFAS or living in regional PFAS hotspots (Chang
et al., 2014; K. T. Eriksen et al., 2009; Lundin, Alexander, Olsen, & Church, 2009; Vieira et al., 2013), particularly in men with familial prostate cancer risk, suggesting a gene–environment interaction (Hardell et al., 2014). Whether PFAS exposures initiate carcinogenesis or promote progression of latent or later stage prostate cancer is unknown for which we hypothesized that PFAS exposure would increase the cell proliferation rate of the prostate cancer cells compared to a non-treated control group (Figure 2). To test the impact of PFAS exposure on prostate cancer initiation, we performed cell viability assays using a benign human prostate cell line, RWPE-1, and a derivative cancerous cell line, RWPE-kRAS (Figure 3A), which showed that PFAS enhances cell viability in an environmentally relevant dose range in both cell types (Figure 3B).

When prostate cancer cells were exposed to varying concentrations of PFAS (10^{-12} M – 10^{-4} M), they exhibited a consequent degree of variance in their rates of proliferation with the first peak in cell proliferation increase observed in 10^{-8} M PFAS, both for PFBS and PFOS. RWPE-kRAS cells showed a more consistent cell proliferation response, with the 10^{-8} M peak being retained. The more aggressive RWPE-kRAS cells exhibited a significant 3.1-fold increase in the rate of cell proliferation when exposed to PFOS (\mu=0.3729, 95\% CI: 0.1155-0.6303, p-val=0.0048, *=p<0.05,**=p<0.01) and a significant 2.9-fold increase in the rate of cell proliferation when exposed to PFBS (\mu=0.3484, 95\% CI: 0.09105-0.6058, p-val=0.0078, *=p<0.05,**=p<0.01) compared to a DHT-treated control group. There was no significant difference between the rates of proliferation of either PFAS compound exposed groups and the DHT-treated control group.

3.3.2. Exposure to PFAS increases the rate of RWPE-kRAS xenograft tumor growth in vivo both in the presence and absence of HF diet.

Compelling evidence from human prostate cell lines and transgenic murine prostate cancer models indicates that a high fat diet contributes to prostate cancer progression by shifting
the prostate metabolome to a pro-cancerous state (Labbé et al., 2019; Priolo et al., 2014). Of note, these actions are mediated through PPARα, the receptor targeted by PFAS, providing potential for synergistic tumor promotion. To test the effects of PFAS exposure, we generated a xenograft tumor growth model on nude immunocompromised mice and injected RWPE cells that have kRAS overexpression ectopically to be able to measure tumor size growth (Figure 4A). At the end of 40 days post-injection, we observed that ectopic tumor volume increased with the fastest rate of growth when PFAS exposure was combined with consumption of HF diet (Figure 4B). This suggests that PFAS exposure in combination with high fat diet consumption likely increases tumor growth more than normal cancer progression. In addition, the rate of growth of the tumors exposed to just PFAS increased compared to the control treated tumor growth rate, which supports the hypothesis that PFAS is detrimental to the prostate cancer progression rate. Finally, we observed that PFAS treatment increased prostate cancer growth rate more than HF diet consumption. As the animals have received slow-release testosterone tube implants, it is likely that the combined PFAS and DHT treatment had a synergistic effect on the tumor growth and a similar effect was also observed in the cell proliferation assays.

3.3.3. Prostate cancer cells that are exposed to PFAS in vitro exhibited increased building block synthesis and altered energy production-adjacent pathways when DHT is present.

Metabolic plasticity is a hallmark of cancer (Hanahan & Weinberg, 2011; Pavlova & Thompson, 2016). Cancer cells respond to their environments by rewiring their metabolic pathways to sustain biological functions. Previous work using transgenic mouse models for Myc-induced prostate cancer found that HFDs increase one-carbon metabolism and associated changes in histone methylation marks, further increasing Myc activity in prostate tumors (Labbé et al., 2019). However, the impact of environmental exposures on prostate cancer cell metabolic
wiring is unknown. Since we observed a synergy between PFOS exposure and high fat diet to increase RWPE-kRAS tumor burden (Figure 4), and an increase in RWPE-kRAS cell viability with PFOS treatment (Figure 3), we performed various –OMICS analysis to study metabolic changes (Figure 5). Prior to our metabolomics, we knew that our prostate cancer cells would proliferate more given a PFAS treatment in the presence of DHT, so we hypothesized that the cell-proliferative energetics pathways would be upregulated in the prostate cancer metabolome. To test this hypothesis, we analyzed metabolites that change in response to PFOS treatment using GC/MS analysis of RWPE-kRAS cell extracts. This analysis showed that PFOS treatment increased the metabolites associated with increased glucose metabolism through Warburg effect, transfer of acetyl groups into mitochondria and citric acid cycle (Figure 6A), particularly pyruvate (Figure 6B). To determine whether the increase in pyruvate production was due to increased expression of enzymes in glycolytic pathway, we performed RNA-seq using tumors from Figure 4. GSEA of the gene expression data identified pyruvate metabolism and glycolysis pathways as significantly upregulated by PFOS exposure in tumors from HFD-fed animals (Figure 6C). Particularly, components of pyruvate dehydrogenase complex (PDC), which is responsible for acetyl-CoA production from pyruvate were increased with PFOS treatment in tumor from high fat diet fed mice (Figure 6D). Consistent with these results, acetyl-CoA level was increased in RWPE-kRAS cells in the conditions where we observed an increase in cell viability (Figure 6E). These results from prostate transformed cell lines show that PFAS exposure increases pyruvate and acetyl-CoA production.

3.3.4. Prostate cancer cells that are exposed to PFAS exhibited increased mitochondrial dependence (citric acid cycle, PPP), increased epigenetic activation (H3K27Ac), acetyl-coA metabolism; and altered amino acid metabolism (serine & lysine) in the absence of DHT.
Like the prior round of GC/MS analysis, the same conditions were tested without the presence of DHT either in the control or the treatment group to see the effects of DHT on the PFAS-prostate cancer paradigm (Figure 7A). As a result, we observed that PFAS treatment upregulated the ‘Threonine & 2-Oxobutanoate Degradation’ (4.757-fold, n=3, p-val=0.000903), ‘Phosphatidylethanolamine Biosynthesis’ (4.057-fold, n=3, p-val=0.0143), ‘Homocysteine Degradation’ (4.036-fold, n=3, p-val=0.0144), ‘Lysine Degradation’ (3.403-fold, p-val=0.0165), – all pathways that are involved in mitochondrial dependence, citric acid cycle regulation and the pentose phosphate pathway (Chen et al., 2021; Leav et al., 2010). Interestingly, biotin metabolism was also significantly upregulated (4.039-fold, n=3, p-val=0.0148), which plays an important role in acetyl-coA carboxylase function as a prosthetic group. These data further reinforced our initial hypothesis that acetyl-coA metabolism is affected with PFAS treatment (Figure 7B, Figure 8).

3.3.5. PFAS treatment increase PPAR signaling and histone acetylation in prostate cancer cells

Based on previously published studies and our data, we hypothesized that in these prostate cells, PFAS converge with a high fat diet to activate PPARα, alter the cell metabolome, and shift carcinogenic risk in normal cells while driving progression in prostate cancer cells. PPARs are transcription factors involved in the regulation of metabolic processes, and HFDs are known to impact hepatic cells through PPARα activation (D. Patsouris, Reddy, Müller, & Kersten, 2006). Our laboratory previously showed that metabolites associated with obesity activate PPARα signaling to modulate ERα activity in breast cancer cells (Madak-Erdogan et al., 2019). It is noteworthy that PFAS chemicals also activate PPARα and, to a lesser extent, PPARβ, to affect metabolism and the immune system (DeWitt et al., 2009). Structurally, PFAS resemble
free fatty acids and bind to the same sites on serum proteins (Evans, 2020.). Published studies show a central role for PPARα signaling in PFOA/PFOS-induced liver and kidney carcinogenesis (Stanifer et al., 2018; Wolf et al., 2008). However, this pathway has not been interrogated for prostate cancer (Figure 9). We hypothesized an upregulation of global gene activity both through epigenetic markers and within other transcriptional factors affect global gene activity. To interrogate transcriptional changes in prostate tumors upon PFAS exposure, we further analyzed our RNASeq analysis. Our data from RWPE-kRAS xenografts show that PFAS exposure combined with high fat diet increases tumor growth and induces transcriptomic changes in PPARα-target genes and genes involved in chromatin organization that in turn regulate transcription (Figure 10A and 10B). In a transgenic MYC model of prostate carcinogenesis, a high fat diet induced changes in H4K20 methylation and impacted expression of MYC-target genes (Labbé et al., 2019). Consistent with these results and earlier changes identified in pyruvate and acetyl-CoA synthesis-related metabolites, we examined the state of a range of histone acetylation and methylation marks in RWPE-kRAS cells exposed to PFOS in HLE and identified significant increases in H3K9 and H3K27 acetylation (Figure 10C). These data support a fundamental role of PPAR signaling and epigenetic changes in prostate cancer xenograft response to PFAS. This suggests that in the presence of PFAS, cellular energetics has shifted from a more proliferative mode to a more mitochondrially dependent mode when testosterone is absent, but further experimentation and repeats should be performed to confirm this emergent pattern.

3.4. DISCUSSION

In the present study, we characterized the effects of PFAS using cell proliferation, metabolomics, metabolite profiling, and western blot assays as well as in vivo xenograft models.
Our cell proliferation and in vivo xenograft model experimental results indicate that prostate cancer cell proliferation rate in vitro was increased nearly 3-fold and the tumor growth rate was faster in the presence of PFAS. According to our metabolomics and metabolite profiling assays, PFAS shifts the cellular energetics of prostate cancer cells, which then increases the rate of proliferation if there is DHT present, but moves the cells to a more energetically efficient and mitochondria dependent state by enhancing oxidative phosphorylation, upregulating pentose phosphate pathway and citric acid cycle.

Combining what has been observed in the metabolomics assay with the fact that testosterone deregulation has increased incidence rates of prostate cancer in men really puts in perspective the critical role that testosterone plays in male reproductive health. Add on top of that the fact that rats treated with testosterone propionate experience a reduction in their prostate cancer tumor size, indicating that proper testosterone concentration maintenance can facilitate tumor prevention in the prostate microenvironment (Umekita, Hiipakka, Kokontis, & Liao, 1996). PFAS exposure seems to interact with the prostate in a similar manner to a testosterone deficient prostate in that it modulates metabolic activity and reduces serum testosterone concentrations, which incidentally a tumor-prone prostate landscape. Further research should be performed to understand whether bioaccumulation of PFAS may lead to similar physiological conditions, and how the liver metabolism deregulation interplays with the aforementioned prostate microenvironment shifts.
CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

4.1. CONCLUSIONS

In this thesis project, we studied the impact of PFAS exposure and high fat diets on prostate carcinogenesis. We found that, PFAS + high fat diet exposure synergized to increase prostate cancer xenograft growth, and PFAS treatment increased glucose metabolism and pyruvate production in prostate cancer cells. Metabolic adaptations in prostate cancer change the epigenetic landscape, in part due to changes in the availability of substrates for epigenetic enzymes. Further, epigenetic marks dictate activity of critical transcription factors for prostate carcinogenesis and progression including PPARα. Despite epidemiological evidence for an association of PFAS exposure with prostate cancer, no mechanistic studies have established the molecular underpinnings whereby PFAS exposures combine with high fat diet to increase prostate carcinogenic risk and promote a more aggressive prostate cancer phenotype.

Epidemiology studies show that prostate cancer risk and mortality increase with PFAS exposure (Barry et al., 2013; Kirsten T. Eriksen et al., 2009; Gilliland & Mandel, 1993) as well as with obesity (Hardell et al., 2014; Vidal et al., 2020). Despite this evidence, mechanistic data on the molecular underpinnings of PFAS chemicals in the prostate are limited/nonexistent. In preliminary studies, we found that PFAS exposure expands the prostate epithelial SPC population, whereas a HFD plus PFAS exposure synergize to increase prostate cancer xenograft growth in mice. Additionally, our results show that PFAS treatment increases glucose metabolism and pyruvate production in prostate cancer cells. It is well-established that metabolic adaptations in prostate cancer alter the epigenetic landscape, in part due to changes in substrate availability for epigenetic enzymes. Further, epigenetic marks dictate the activity of PPARα, a
critical transcription factor in PFAS-associated carcinogenesis (Stanifer et al., 2018; Wolf et al., 2008) that is liganded by both PFAS (Evans, 2020) and metabolites associated with HFDs (David Patsouris et al., 2006).

We showed that metabolic alterations from high fat diets combined with PFAS exposures play a significant role in prostate cancer initiation and tumor progression. Published studies clearly demonstrate that metabolic changes impact epigenetic marks during tumor progression (Deblois et al., 2020; Makohon-Moore et al., 2017; McDonald et al., 2017). Metabolic alterations in cancer cells result in epigenetic reprogramming due to changes in the availability of substrates for epigenetic enzymes (Boukouris, Zervopoulos, & Michelakis, 2016; Faubert, Solmonson, & DeBerardinis, 2020; Pascual, Domínguez, & Benitah, 2018). Local acetyl-CoA production, via recruitment of metabolic enzymes to chromatin, enables coordination of environmental cues with histone acetylation and gene transcription, which increases fitness and survival of cancer cells. Acetyl-CoA can be synthesized in the nucleus from pyruvate by the pyruvate dehydrogenase complex (PDC) (Matsuda et al., 2016; Sutendra et al., 2014), from acetate by acetyl-CoA synthetase 2 (ACSS2) (Li, Qian, & Lu, 2017; Li, Yu, et al., 2017), and from citrate by ATP-citrate lyase (ACLY) (Wellen et al., 2009) (Figure 11).

Overall, our studies suggest that PFAS exposure through oral ingestion can lead to an increased risk of prostate cancer incidence. This increased incidence risk of prostate cancer through PFAS exposure prompted the investigation of various nuclear receptors and mechanisms of induction. PPARα, a nuclear receptor that can induce the carcinogenesis and proliferation of prostate cancer, was found to be activated by various types of PFAS. PFOS and PFOA were two specific chains that showed a higher level of activation of PPARα compared to the other types that researched in this investigation. In addition, the deregulation of liver metabolism through
PFAS exposure seems to have potential to develop non-alcoholic fatty liver disease or other metabolic disorders. Finally, the interactions between PFAS and testosterone metabolism can lead to a reduction in serum testosterone concentration which can increase chance of prostate cancer development.

4.2. FUTURE DIRECTIONS

Additional research on the other mechanisms and receptors that induce prostate cancer through PFAS exposure must be conducted to help pinpoint the specific substances that can be used as an alternative to PFAS in various high use frequency avenues such as firefighting foams, grime-resistant Teflon pans and water pipes to avoid triggering these oncogenic pathways. Research in this field is necessary to help protect the health and well-being of populations who are exposed to high levels of PFAS, such as firefighters. Researchers and manufacturers must work together to develop substances that replicate the purpose of PFAS in firefighting foams, but that do not pose a substantial risk to firefighters.

Further investigation of the specific types of cancers associated with PPAR activation should be conducted to determine the link between specific PFASs on types of cancers with PPAR being a factor of correlation. Understanding individual types of PFAS activation on PPARα, β/δ, and γ can provide insight for selecting the proper PFAS to be used in firefighting foams that can prevent specific types of cancers.
FIGURES AND TABLES

Figure 1. Thesis summary
Determine the effects of PFAS on prostate cancer proliferation & disease prognosis

Hypothesis 1: Prostate cancer cells exposed to perfluorooctane sulfonate (PFOS; a type of PFAS compound) have altered degrees of cell viability & proliferation in vitro.

Hypothesis 2: Prostate cancer xenograft tumors, when exposed to PFOS, have altered growth rates in vivo.

Figure 2. Hypothesis and methods for studying PFAS impact on prostate cancer proliferation
Figure 3. PFAS treatments increase viability of prostate benign (RWPE-1) and cancerous (RWPE-kRAS) cells. A. Experimental design. B. Cells were treated with indicated doses of PFAS for 1 week. Cell viability was measured using WST1 assay.
**Figure 4.** PFAS and HFDs synergize to increase prostate cancer xenograft growth. A. Experimental design. B. $10^6$ RWPE-kRAS cells were injected subcutaneously in 4 week old athymic nude male mice. Mice were fed a high fat diet (HDF) and treated with 10 µg/kg PFOS for 5 days/week orally. Tumor volume was measured using an electronic caliper three times/week. C. For the duration of the 40-day treatment regimen, animal weight steadily increased, but did not vary significantly between different treatment groups. Body weight was measured every 3 days. D. Food consumption between different groups did not significantly vary. Both high fat and Western control diets were varied in terms of how much each group has consumed throughout the week, depending on how many days elapsed since the last day of measurement, but the amount of consumption did not vary between different treatment groups.
Determine the impact of PFAS exposure on prostate cancer metabolism

**Hypothesis 3:** Prostate cancer cells exposed to perfluorooctane sulfonate (PFOS; a type of PFAS compound) will shift the way in which they uptake and utilize metabolites like acetyl Coenzyme-A and pyruvate in vitro.

**Hypothesis 4:** The treatment of PFOS on prostate cancer cells will also change the activity of many gene regulatory elements and subsequently the genes they affect in vitro.

**Hypothesis 5:** Due to the change in metabolite uptake exhibited in different treatment groups, we will also see a change in the metabolite concentrations (Acetyl Coenzyme-A & Pyruvate) present in the cells when the metabolites are visualized and quantified fluorescently.

Figure 5. Mechanistic studies to understand molecular basis of PFAS induces prostate carcinogenesis. Hypothesis and methods used are highlighted.
Figure 6. PFAS treatment increases pyruvate and acetyl-CoA levels in RWPE-kRAS cells.

A. PFOS-induced metabolites in RWPE-kRAS cells identified by GC/MS analysis. B. Pyruvate levels from A. C. GSEA of PFOS+HFD induced genes in RWPE-kRAS xenografts identified by RNA-Seq analysis. D. mRNA expression of PDHB and PDHX, components of PDC, were increased with PFOS and high fat diet in RWPE-kRAS xenografts. E. Acetyl-CoA levels in PFOS-treated RWPE-kRAS cells (10 nM PFOS ± DHT, 24 hr) using a fluorescence based assay. *p<0.05, **p<0.01.
Figure 7. PFAS treatment increases betaine metabolism, which can be associated as a metabolic side-effect of PFAS exposure in RWPE-kRAS cells. A. PFOS-induced metabolites in RWPE-kRAS cells identified by GC/MS analysis. Individual metabolic pathways that were in the top 25 of the upregulated pathways were analyzed for significance and enrichment ratio. The only significantly upregulated pathway was betaine metabolism ((p-val=0.00819, ER=4.097) and the rest of pathways were upregulated, but were not statistically significant. B. The top 25 metabolic pathways that were upregulated are shown with varying degrees of significance (p-val range: 0.008-0.3)
Figure 8. PFAS exposure upregulates mitochondrial dependence (citric acid cycle, PPP), increased epigenetic activation (H3K27Ac), acetyl-coA metabolism; and altered amino acid metabolism (serine & lysine) in the absence of DHT. Metabolic pathways that were upregulated in the PFAS-treated group compared to a non-treated control group were cross referenced and pathways that were upregulated in the PFAS group were listed based on enrichment ratio.
Hypothesis 5: Histone modifications, when PFAS is introduced, shifts in a way that increases global gene activity in many epigenetic regulation loci in vitro.

Figure 9. Outline of studies to understand the impact of PFAS on epigenetic regulation in prostate cancer cells.
Figure 10. PFAS treatment increases A PPAR signaling, B epigenetic regulation of transcription-associated genes in RWPE-kRAS xenografts. C PFOS exposure increased histone acetyl markers in RWPE-kRAS cells.
Figure 11. Link between metabolic pathways and epigenetic marks.


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