THE IMPACT OF PERFLUOROALKYL SUBSTANCES ON THE CELLULAR UPTAKE OF THYROID HORMONES

By

JULIAN SELANO

(Under the Direction of Jason Zastre)

ABSTRACT

Thyroid hormones (TH) are endogenous compounds critical to mammalian metabolic regulation. Perfluoroalkyl substances (PFASs) are a group of xenobiotics known to influence the systemic concentration on THs *in vivo*, however their mechanism in not completely elucidated. The purpose of this work was to investigate the impact of two widely distributed PFAS compounds, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS), on TH uptake as a potential mechanism of TH disruption. Using cryopreserved rat hepatocytes, we demonstrate that PFOA and PFOS are able to increase uptake of the TH, thyroxine (T_4), as a consequence of competitive displacement from the serum binding protein transthyretin. To further assess the impact of hepatic transporters on T4 uptake, we evaluated T4 transport in liver specific organic anion transporting polypeptides (OATPs). Interestingly, transport of T4 was demonstrated in rat Oatp1b2 but not in human OATP1B1. Overall these findings provide a mechanism

by which PFAS compounds may facilitate a decrease in systemic TH concentrations in vivo.

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B.S., Pharmaceutical Sciences, University of Georgia, 2016

A Thesis Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

Athens, Georgia

2018

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December 2018

ACKNOWLEDGEMENTS

I'd like to thank all of those who have helped me reach this goal. First and foremost, I'd like to thank my family for their unending support. Their encouragement has been paramount to my success.

I'd also like to thank my friends for their encouragement and company. Without that time to relax and unwind, I don't know that I would have had the energy to get through this.

To my current and past lab mates, I'd like to thank you for your help with research and coursework, as well as providing encouragement through the years. Kristy and Hunter, thank you both for your time putting up with my mistakes and helping me become the researcher I am today.

Chris Mazur, thank you for taking a chance on me and working with me over the last few years to develop my understanding of science and research. My time and the E.P.A. will act as a springboard for my science career and the work you have done to help me will be a great foundation for my future.

To my committee members, thank you for your time and expertise over the last few years.

And a final thank you to Dr. Zastre. Words cannot express my gratitude to you for the guidance, encouragement and expertise you have provided to me. I have seen myself grow as both a person and researcher since joining your lab you have played a very large role in that.

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ABBREVIATIONS

ADHD: Attention deficit hyperactive disorder

BBB: Blood brain barrier

CSF: Cerebrospinal Fluid

DIO: Deiodinase

FT3: Free T3

FT4: Free T4

HPT axis: Hypothalamic-Pituitary-Thyroid axis

LAT: L-type amino acid transporter

MCT: Monocarboxylate transporter

MRP: Multidrug resistance-associated protein

NHANES: National Health and Nutrition Examination Survey

NTCP: Sodium taurocholate co-transporting polypeptide

OAT: Organic anion transporter

OATP: Organic anion transporting polypeptide

PFAS: Perfluoro alkyl substance

PFCA: Perfluoro carboxylic acid

PFDoDA: Perflurododecanoic acid

PFNA: Perfluorononanoic acid

PFOA: Perfluorooctanoic acid

PFOS: Perfluorooctane sulfonic acid

PFSA: Perfluoro sulfonic acid

PFUnDA: Perfluoroundecanoic acid

rT_3: Reverse triiodothyronine

SA: Serum Albumin

SULT: Sulfotransferase

T_2: Diiodothyronine

T_3: L-3,5,3'-triiodothyronine

T_4: L-3,5,3',5'-tetraiodothyronine (thyroxine)

TBG: Thyroid Binding Globulin

Tg: Thyroglobulin

TH: Thyroid Hormone

TPO: Thyroid Peroxidase

TR: Thyroid receptors

TRH: Thyrotropin-releasing Hormone

TSH: Thyroid Stimulating Hormone

TT_3: Total T_3

TT_4: Total T_4

TTR: Transthyretin

UGT: UDP-glucuronosyltransferase

DISCLAIMER:

Experimental results found herein were collected at the U.S. Environmental Protection Agency under the supervision of Chris Mazur (Athens, Ga/ORD/NERL) and funded through an Oak Ridge Institute of Science and Education fellowship. The views expressed in this work are those of the author and do not necessarily represent the views or polices of the U.S. EPA.

CHAPTER 1

Introduction and Literature Review

1.1 Thyroid Hormones

Thyroid hormones (THs) are endogenous compounds critical to metabolism, growth, and development in the mammalian body. Their functions include influencing heart rate, core temperature, lipolysis, gluconeogenesis, differentiation of neurons, and creating new neural networks. As a result of their importance to many different organ systems, even slight disruption in the systemic levels of THs can result in serious health consequences (Mullur *et al.* 2014). Thyroid dysfunction is reported to be prevalent in up to 10% of the world's general population (Screening 2003). Among its underlying etiologies, xenobiotic exposure is demonstrated to play a role in causing thyroid dysfunction (Haddad 2008). The mechanisms by which some xenobiotics cause a negative impact on THs is not completely understood. Developing a mechanistic understanding of these impacts could improve future risk assessment for xenobiotic exposures.

1.1.1 Thyroid hormone production and regulation

THs are regulated by a negative feedback system called the hypothalamic-pituitary-thyroid axis (HPT axis) (Mondal et al. 2016). As the name suggests, this negative feedback mechanism involves signaling between the hypothalamus, pituitary, and thyroid gland to either produce or cease secretion of thyroxine (T_4). Feedback is initiated in the hypothalamus following binding of triiodothyronine (T_3) to target receptors in the nucleus. A decrease in serum TH levels signal for a release of thyrotrophin-releasing hormone

(TRH). TRH can be detected by a G-protein coupled receptor in the pituitary, leading to production and secretion of thyroid stimulating hormone (TSH) (Mondal et al. 2016, Costa-e-Sousa & Hollenberg 2012). TSH then binds to a G-protein coupled receptor in the thyroid gland and begins a signaling pathway resulting in the production of the sodium iodide symporter (NIS), thyroglobulin (Tg), and thyroid peroxidase enzymes (TPO) critical to TH production (Goel *et al.* 2011).

Production of thyroid hormones begins in the thyroid gland following with the uptake of systemic dietary iodide into thyroid follicular cells by the NIS transporter upregulated during TSH stimulation. Intracellular free iodide is subsequently incorporated on tyrosyl residues of the protein thyroglobin (Tg) by the thyroid peroxidase (TPO) enzyme. Following this reaction, TPO joins two iodotyrosyl residues by phenolic coupling to create the prohormone T_4 (Fig. 1.1). Proteolytic cleavage of T_4 from Tg releases the thyroid hormone and allows for hormone secretion. The process of proteolytic cleavage also creates a relatively small amount of biologically active T_3 (Fig. 1.1). Following release, free T_4 and T_3 are secreted into the blood stream where T_4 makes up the predominant fraction of TH in serum (80%) (Mondal et al. 2016).

Thyroid Hormones Produced by the Thyroid Gland

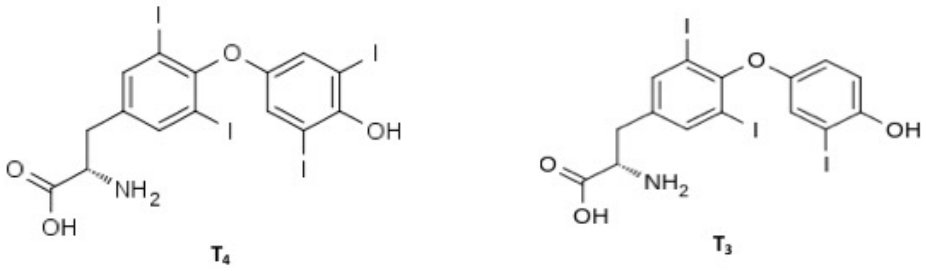

Figure 1.1: Thyroid Hormones Produced and Released by the Thyroid Gland. (T_4) L-3,5,3',5'-tetraiodothyronine. (T_3) L-3,5,3'-triiodothyronine

1.1.2 THs bound in serum

Following secretion from the thyroid gland, THs bind to proteins in serum, which act as a buffer system to maintain a stable free hormone fraction and improve even distribution of THs (Mendel 1989, Palha *et al.* 1994, Mondal et al. 2016). It is estimated that approximately 99.7% of THs found in serum are bound to the following 3 major TH binding serum proteins in nearly all vertebrate species: thyroid binding globulin (TBG), transthyretin (TTR), and serum albumin (SA) (Mondal et al. 2016, Refetoff 2000). TBG has the highest affinity for THs and acts as the primary TH binding protein in human serum, binding 75% of T_4. TTR has the second highest affinity for THs and ranks as the intermediary TH binding protein in humans, binding 20% of T_4 in serum. SA demonstrates the weakest affinity for T_4 systemically, and accounts for approximately 5% of T_4 binding in humans (Table 2) (Refetoff 2000) .

Table 1.1: Relative T4 binding, abundance and T_4 association constant of thyroid binding serum proteins: thyroxine binding globulin (TBG), transthyretin (TTR), serum albumin (SA). Table adapted from Reffetoff *et al*., 2000.

Thyroid Binding Serum Proteins

	Relative T_4 binding in serum	Abundance in Serum (mg/L)	T_4 Association Constant (Ka)
TBG	75%	16	1.0×10^{10}
TTR	20%	250	2.0×10^{8}
SA	5%	40,000	1.5×10^{6}

TBG is the predominant TH binding protein in serum despite being the least abundant. A single TH binding site is present on a TBG molecule for the binding of T_3 or T_4 (Refetoff 2000). TTR presents two potential binding sites to T_4, however due to negative cooperativity, only one binding site is occupied at a time. TTR also presents a unique function as the main TH binding protein in cerebrospinal fluid (CSF). It is estimated that TTR is responsible for binding approximately 85% of T_4 in the CSF, highlighting an important role in distributing T_4 to neurons (Richardson *et al.* 2015). SA presents three different binding sites to both T_4 and T_3, however the affinity for SA is much less than that of other serum proteins. As such SA acts as the least abundant binding protein for THs despite its prevalence in human serum (Refetoff 2000).

1.1.3 TH uptake

Uptake of TH is a rate-limiting step of TH activation, action, and excretion as TH conversion occurs via intracellular mechanisms (Hennemann *et al.* 2001). THs can enter a cell via two processes, simple passive diffusion and carrier-mediate transport (Fig. 1.2). Due to the hydrophobic nature of T_4, it was historically believed that THs entered the cell purely via passive diffusion. However, *in vitro* studies have characterized transporters for TH uptake (Hennemann et al. 2001, Visser et al. 2011). These transporters include members of the L-type amino acid transporter (LAT), organic anion transporting polypeptide (OATP), organic anion transporter (OAT), sodium taurocholate co-transporting polypeptide (NTCP), and monocarboxylate transporter (MCT) families (Table 1.2) (Visser et al. 2011, Friesema *et al.* 2012).

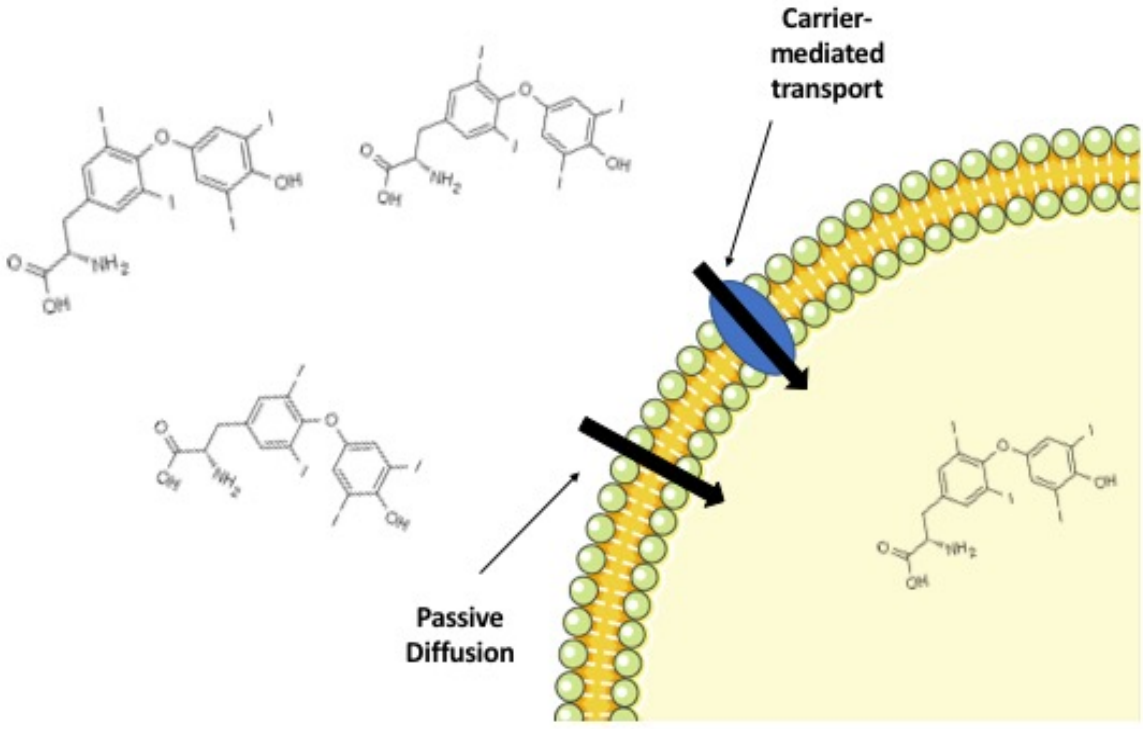

Figure 1.2: Mechanisms of T_4 Uptake. Figure demonstrates T_4 uptake via carrier-mediated transport and passive diffusion.

Of the TH transporters currently identified, some are noted for their particular importance due to their substrate specificity and tissue expression. MCT8 is a member of the MCT family with a very high affinity and specificity for THs (Friesema et al. 2012). These transporters are also predominantly expressed in neurons and the liver; locations being important for both TH function and TH clearance, respectively (Friesema *et al.* 2003). *In vivo* and epidemiological analyses have found that genetic mutations in MCT8 can have drastic impacts on TH uptake and intracellular activity, leading to symptoms similar to that of hypothyroidism (Visser et al. 2011). OATP1C1 plays an important role in TH uptake at the BBB, where TH transport to the CSF is critical to maintaining neuronal function and promoting development (Visser et al. 2011, Friesema et al. 2012, Mayerl *et al.* 2012). The OATP1B and OATP2B subclasses are also important for TH uptake, as they are the most abundant group of transporters in the liver (Burt *et al.* 2016). Interestingly, the liver has a multitude of different transporters known to transport TH. Of note, OATP1B1, OATP1B3, OATP2B1, MCT8, and NTCP can all be found on the basolateral membrane of hepatocytes (Burt et al. 2016, Friesema et al. 2003). The presence of this multitude of transporters allows for extensive TH liver uptake, leading to both metabolic activation as well as T4 clearance and excretion.

Table 1.2: Previously characterized transporters of thyroid hormones. Capitalized names are human transporters, lowercase titles are animal transporters. Table adapted from Visser *et al*., 2011

Thyroid Hormone Transporters	
Transporter	**Characterized substrate**
Oatp1a1	T_4, T_3, rT_3, T_2, T_4S
OATP1A2	T_4, T_3, rT_3
OATP1B1	T_4, T_3, T_3S, T_4S
Oatp1b2	T_4, T_3
OATP1B3	rT_3, T_4S, T_3S, rT_3S
OATP1C1	T_4, rT_3, T_3, T_4S
Oatp4a1	T_4, T_3, rT_3
OATP4C1	T_4, T_3
MCT8	T_4, T_3, rT_3, T_2
MCT10	T_4, T_3
LAT1	T_4, T_3, rT_3, T_2
LAT2	T_4, T_3, T_4S, T_3S
NTCP	T_4, T_3, T_4S, T_3S

1.1.4 Metabolism of TH

Following uptake of into local tissues, THs are subject to a variety of metabolic reactions. These metabolic pathways can lead to activation or deactivation of THs that result in the creation of functional T_3 and a reservoir of inactive TH metabolites (van der Spek *et al.* 2017, Mondal et al. 2016). This reservoir of TH metabolites is presumed to play a functional role in maintaining nuclear receptor sensitivity to THs, conserving free iodide, and in some cases acting as reserve for later TH conversion (van der Spek et al. 2017). The following sections detail the metabolic pathways that create functional THs and their inactive metabolites.

1.1.4.1 Metabolic activation of THs

Activation of THs is considered to be the conversion of THs to the T_3 form. The most common activation is the conversion of T_4 to T_3. This reaction is mediated by two members of the deiodinase enzyme family, Type I (DIO1) and Type II (DIO2) (van der Spek et al. 2017). The most important is the DIO2 isoform responsible for converting the majority of T_4 to T_3 by removing an iodine molecule from the outer phenolic tyrosyl ring (Fig. 1.3) (van der Spek et al. 2017). DIO1 is also responsible for converting T_4 to T_3, however the substrate affinity of DIO1 is much greater for reverse T_3 (rT_3) and conjugated metabolites, and is therefore mainly viewed as an inactivation pathway (van der Spek et al. 2017). Hydrolytic cleavage of previously conjugated T_4 and T_3 can also occur, rendering either functional T_3 or T_4 that is subsequently converted to T_3 (Fig. 1.3) (Mondal et al. 2016).

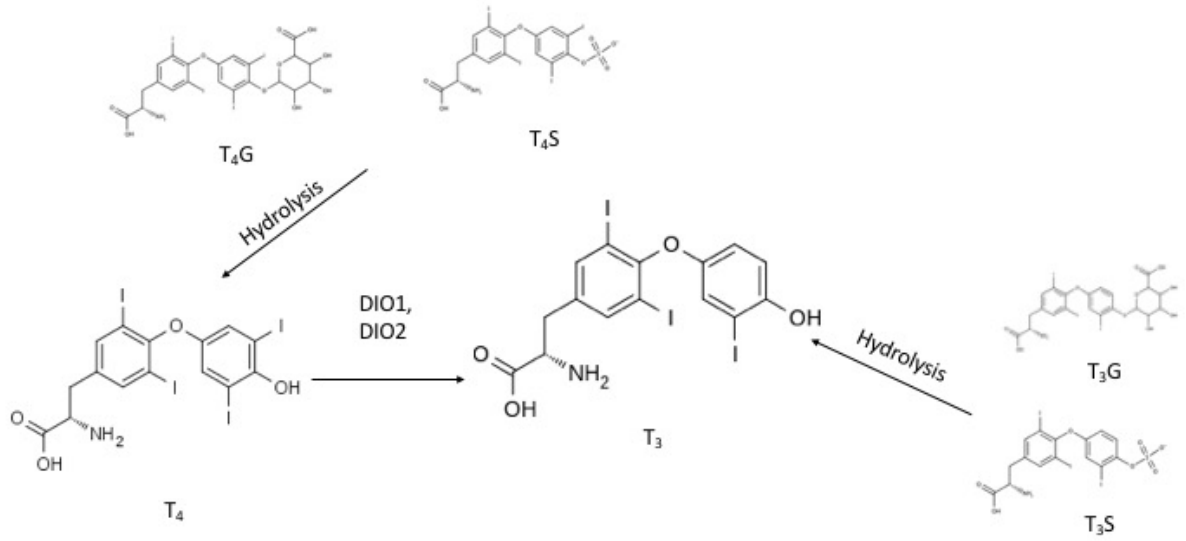

Figure 1.3: Metabolic Activation of T_3 from T_4 and TH Conjugates. Main TH conversion is thyroxine (T_4) to triiodothyronine (T_3) by deiodinase type I (DIO1) and type II (DIO2). TH glucuronide (T_4G, T_3G) and sulfate (T_4S, T_3S) conjugates undergo hydrolysis to revert to parent forms.

1.1.4.2 Metabolic deactivation of THs

Deactivation of THs occurs primarily as conversion of T_4 and T_3 to a variety of different forms based on the metabolic enzyme. The most prominent inactive metabolites of T_4 and T_3 are reverse-T_3 (rT_3) and diiodothyronine (T_2). Both reactions occur via deiodinase enzymes. DIO1 and DIO3 isoforms are capable of inner-ring deiodination, responsible for converting T_4 to rT_3 and T_3 to T_2, respectively. Outer-ring deiodination by DIO2 is also capable of converting rT_3 to T_2 (Fig. 1.4) (van der Spek et al. 2017). Conjugation of THs can add to the available reserve of TH metabolites, however this metabolic conversion also makes THs more prone to elimination. Therefore, this pathway is useful in regulating TH levels by increasing TH clearance. Conjugation occurs by common sulfotransferase (SULT) and UDP-glucuronosyltransferase (UGT) enzymes. Of these enzyme families, only SULT1 is able to convert T_3 and T_4 to their respective sulfated conjugates (T_3S and T_4S), and UGT1A9, 2B7, and 1A1 are able to conjugate T_4, T_3, and rT_3 with a glucuronide functional group (Fig. 1.4) (Mondal et al. 2016).

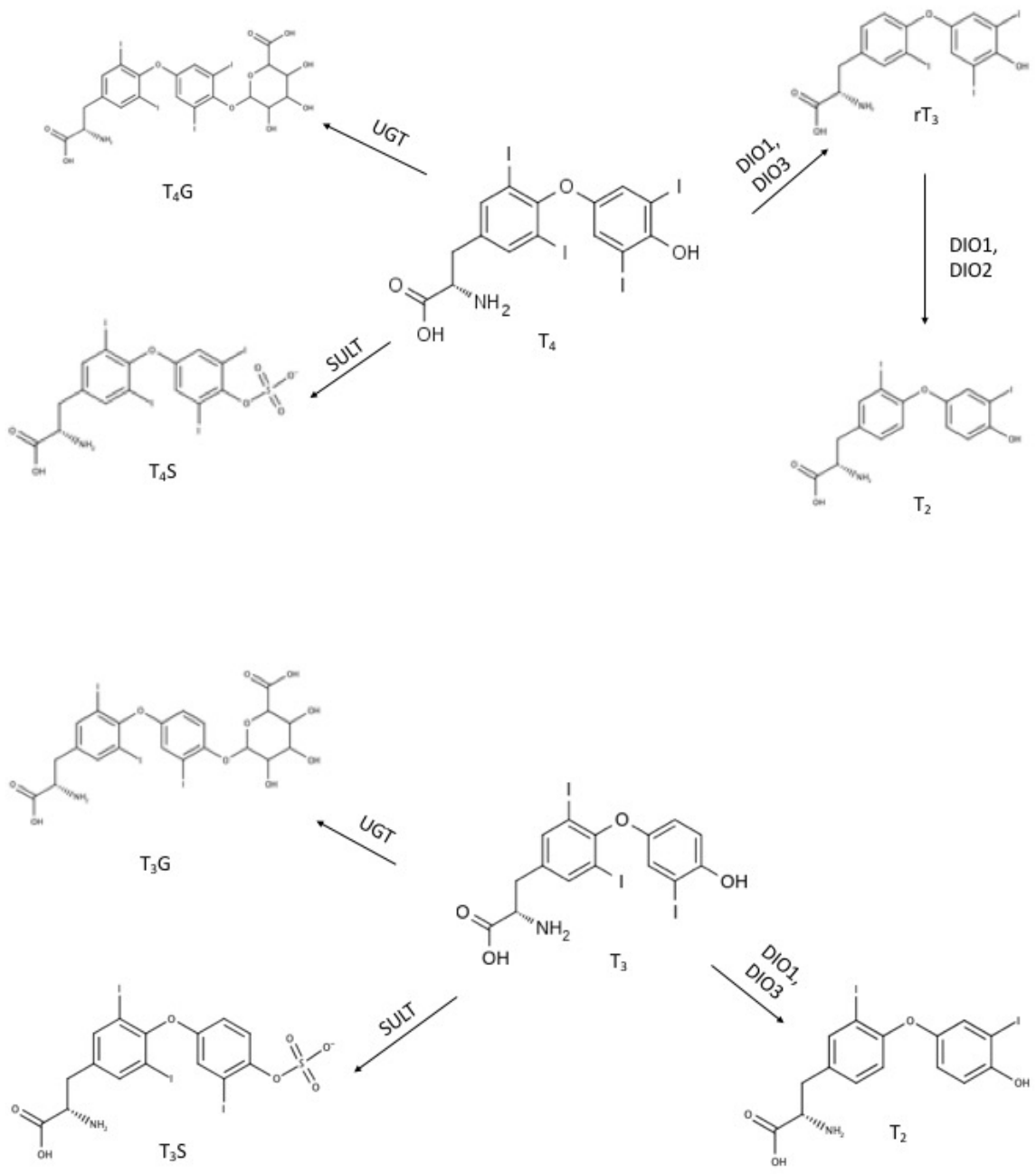

Figure 1.4: Metabolic Deactivation of Thyroid Hormones T_3 and T_4 by sulfotransferases (SULT), UDP-glucuronosyltransferases (UGT), and Deiodinase Types I (DIO1), II (DIO2), and III (DIO3).

Certain tissues are known to preferentially express various TH metabolic enzymes due to specific need or function. DIO1, SULT1, and UGTs are expressed to a higher extent in the liver and kidney. This is a result of liver and kidney function as excretory organs responsible for TH clearance. Conversely, tissues such as skeletal muscle, cardiac muscles, and neurons preferentially express high levels of DIO2, as there is a greater need for active T_3 in these tissues (van der Spek et al. 2017). Expression of DIO2 and DIO3 is especially critical to neuronal function as the cerebral cortex relies on 80% of its nuclear T_3 from intracellular conversion of T_4 (van der Spek et al. 2017).

1.1.5 TH function

After conversion of THs to the biologically active form, T_3 enters the nucleus and binds to thyroid receptors (TRs) α and β. These TRs then dimerize with other nuclear receptors, before binding to thyroid response elements of the promoter region of the DNA sequence to encode transcription of enzymes critical to metabolism, growth, and development (Mondal et al. 2016, Liu & Brent 2010). THs have a profound impact on function of the cardiovascular system, the reproductive system, the skeletal system, and the brain (Fig. 1.5) (Mondal et al. 2016). Notably, THs have a significant role in neuronal development and stability. New neurons need TH in order to properly differentiate and create new neural networks (Moog *et al.* 2017, Calza *et al.* 2015, Remaud *et al.* 2014). Lack of TH is often associated with depression, decreased cognitive function, and memory loss.

THs also play a critical role in developing children (Moog et al. 2017, Gilbert *et al.* 2012). During pregnancy, fetal production of THs does not start until the third trimester (Gilbert et al. 2012). Therefore, the fetus is dependent upon the mother's circulating THs

to begin the delicate steps of neurogenesis and differentiation. As a result, a lack of TH during developmental stages of maternity have been associated with a host of significant ailments (Moog et al. 2017, Gilbert et al. 2012). The most devastating of which is the irreversible damaged caused to the brain, resulting in higher risk of autism, ADHD, and decreased motor function (Roman *et al.* 2013, Henrichs *et al.* 2013, Li *et al.* 2010, Pop *et al.* 2003, Gyllenberg *et al.* 2016).

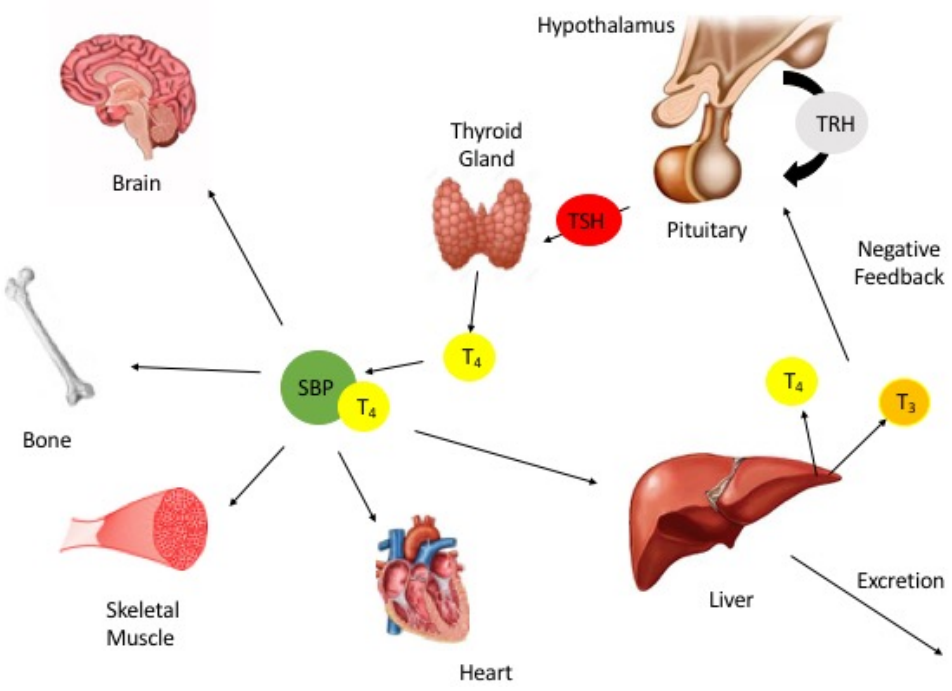

Figure 1.5: Schematic of Thyroid Hormone Function. Abbreviations: serum binding proteins (SBP), Thyrotropin releasing hormone (TRH), Thyroid stimulating hormone (TSH). Adapted from Mondal *et al.,* 2016

1.1.6 Thyroid Hormone Related Diseases

Significant alterations in the circulating TH levels often lead to a host of health impacts that vary depending on hyper or hypothyroid states. The most critical impacts of changing systemic TH levels are on cardiovascular health, mental health, and weight

change (Mondal et al. 2016). These effects can be a result of malformation of the thyroid gland (thyroid cancer or goiter), improper dietary iodine intake, autoimmune disease, or xenobiotic exposure (Haddad 2008). Changes in thyroid hormone are clinically screened by measuring TSH levels and further evaluated by measuring serum total and free T_4 and T_3. Diagnosis is then achieved by comparing the results to reference ranges of healthy individuals (Table 1.3) (Van Vliet & Deladoey 2014). Variations in the degree of changes in TH levels leads to different classifications of TH disease states (Vanderpump 2011). The impacts and further definition of each thyroid disease state classification will be discussed in the following sections.

Table 1.3: Reference Intervals for TH Levels of Subjects Considered to be Healthy. Table adapted from Kratzsch *et al.,* 2005

Thyroid Hormone Reference Intervals

Hormone	Median	2.5^{th} - 97.5^{th} percentiles
TSH (mIU/L)	1.36	0.4 – 3.77
TT_4 (nmol/L)	98.3	70.5 - 157
TT_3 (nmol/L)	1.77	1.27 – 2.79
FT_4 (pmol/L)	16.2	12.8 – 20.4
FT_3 (pmol/L)	5.13	4.02 – 6.79

1.1.6.1 Hyperthyroidism

Incidence of hyperthyroidism in iodine replete communities is stated to be between 0.5-2% in women, with hyperthyroidism being 10 times more prevalent in females than males (Vanderpump 2011). Two major classes of hyperthyroidism are generally identified clinically. Overt hyperthyroidism is defined as low serum TSH (< 0.1 mIU/L) and higher

serum total T_4 concentration (> 170 nmol/L) (Hollowell *et al.* 2002). Subclinical hyperthyroidism is defined as low serum TSH (< 0.4 mIU/L) and normal serum total T_4 (Hollowell et al. 2002). Subclinical hyperthyroidism is generally considered to be a milder form of hyperthyroidism and is often a sign of progression towards overt hyperthyroidism. Clinical manifestations of hyperthyroidism include tachycardia, systolic hypertension, heat intolerance, ophthalmopathy, menstrual disturbances, tremors, weight loss, and muscle weakness (Haddad 2008). The most common causes of hyperthyroidism are Graves' disease (autoimmune disease), followed by toxic multinodular goiter (Vanderpump 2011).

1.1.6.2 Hypothyroidism

Incidence of hypothyroidism is stated to be 3-5% of the general population of iodine replete communities, with prevalence above 10% in cohorts of elderly women above the age of 45 (Vanderpump 2011, Haddad 2008). Much like hyperthyroidism, the prevalence of hypothyroidism in women is roughly 10 times that found in men (Vanderpump 2011). Overt hypothyroidism is defined by elevated serum TSH and low serum free T_4 concentrations. Subclinical hypothyroidism is described as elevated serum TSH levels with free T_4 concentration within the nominal reference range (Haddad 2008). Much like hyperthyroidism, subclinical hypothyroidism is considered a milder form of hypothyroidism and a sign of progression towards overt hypothyroidism. Unlike hyperthyroidism, a third classification for hypothyroidism exists. Hypothyroxinemia is classified as serum TSH within reference range and reduced free T_4 serum concentrations (<2.5th percentile) (Negro *et al.* 2011). This form of hypothyroidism is generally diagnosed during pregnancy and is linked to many adverse health effects on

the developing fetus (Negro et al. 2011). Other clinical manifestations of hypothyroidism include bradycardia, fatigue, cold intolerance, weight gain, dry skin, edema, depression, mental impairment, slow movement, decreased appetite, and impaired visual field (Haddad 2008). The most common causes of hypothyroidism are Hashimoto's thyroiditis (autoimmune disease), thyroidectomy, and insufficient iodine intake in iodine depleted communities (Vanderpump 2011).

While not generally considered a predominant underlying etiology, xenobiotic exposure is still identified as a cause of both hyper and hypothyroidism (Haddad 2008). Both drug products and environmental contaminants have been identified as having negative impacts on TH serum concentrations and TH action. For example, Amiodarone is an iodine rich drug product that is capable of inducing hyperthyroidism, and has also known to increase severity of hypothyroidism in those previously afflicted with Hashimoto's thyroiditis (Harjai & Licata 1997). Lithium salts have led to the development of hypothyroidism in over 20% of patients after 10 years of therapy (Perrild *et al.* 1990). Tyrosine kinase inhibitors used in the treatment of cancer also demonstrate development of hypothyroidism in up to 36% of patients (Desai *et al.* 2006). Additionally, synthetic pollutants including pesticides and herbicides have demonstrated an ability to induce hypothyroidism (Brucker-Davis 1998). Little is known about the mechanism by which these xenobiotics are able to disrupt THs. It is thought that this action may be a function of disrupting TH nuclear receptor binding, plasma protein binding, TH distribution, or TH clearance (Gilbert et al. 2012). More research is needed to evaluate the impact of xenobiotic exposure on exogenous THs (Brucker-Davis 1998).

1.2 Perfluorinated alkyl substances

Perfluorinated alkyl substances (PFASs) are a class of xenobiotic compounds known to have an impact on physiological T_4 levels (Chang *et al.* 2008, Coperchini *et al.* 2017). They are characterized by having per- or poly-fluorinated alkyl chains of varying length, and a charged functional end group (fig. 1.6) (Krafft & Riess 2015). These chemical characteristics give PFASs unique surfactant properties that create an amphiphilic nature, as well as advantageous stability and fluidic properties. This makes PFASs appealing for a wide variety of commercial and industrial applications, such as use in fire-fighting foams, automotive and avionic hydraulics, food packaging, water and stain resistant textiles, and photographic imaging (Krafft & Riess 2015). Extensive PFAS use has led to widespread distribution and contamination of soil and water causing fairly ubiquitous human exposures (Nguyen *et al.* 2016, Perez *et al.* 2013, Liu *et al.* 2015). The impact of PFASs on THs has been well demonstrated, however the exact mechanisms of PFASs effect on TH are not completely understood (Coperchini et al. 2017).

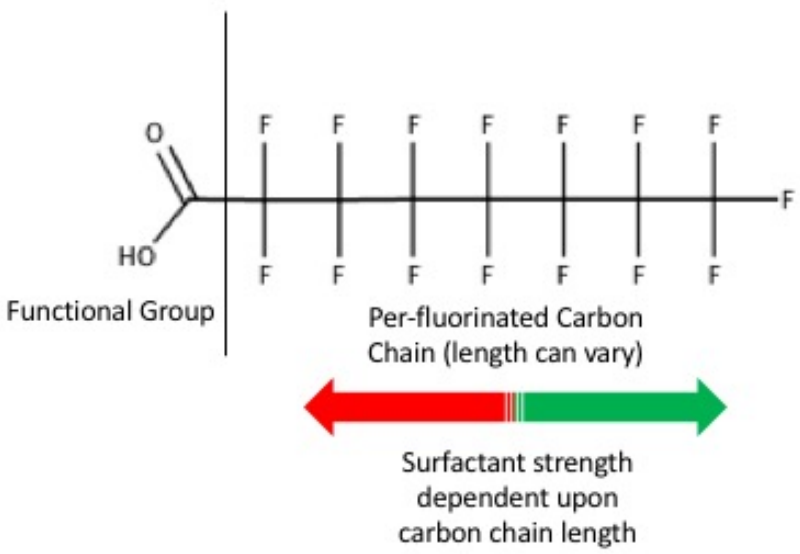

Figure 1.6: Illustration of PFAS Structural Components. Surfactant strength and physicochemical properties of PFAS are increased as the carbon chain length increases. The inverse is true for decreasing carbon chain length.

1.2.1 PFAS structure and uses

Poly- and per-fluoro alkly substances can be divided into two categories consisting of short chain perfluorinated surfactant (fluorosurfactants) or perfluorinated polymers (Krafft & Riess 2015). While perfluorinated polymers play an important role in consumer products, monomer fluorosurfactants are more biologically active, and therefore present a greater potential risk to human health (Krafft & Riess 2015). Perfluoro carboxylic acids (PFCAs) and perfluorosulfonic acids (PFSAs) are the largest families of monomer fluorosurfactants (Fig. 1.7) (Prevedouros *et al.* 2006). Among these families, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) are the most widely distributed compounds (Calafat *et al.* 2007, Frisbee *et al.* 2009). The combination of the highly hydrophobic fluorocarbon chain and the highly hydrophilic functional group combine to create exceptional surface properties unmatched by most other organic

surfactants (Krafft & Riess 2015). The strength and stability of the C-F and C-C bonds of the alkyl chain also provide resistance to UV radiation and thermal degradation (Krafft & Riess 2015). In a study conducted on fluorosurfactants with sulfonic acid and carboxylic end groups, it was found that these compounds are stable up to 400^{o}C, demonstrating the intense thermal stability PFAS compounds are able to achieve (Krafft & Riess 2015). These compounds are also extremely inert as the size of the fluorine atoms are thought to act as a shield to chemical attack of the carbon backbone. The properties contained by perfluorosurfactants are largely dependent upon the length of the perfluorinated alkyl chain. An increase in chain length creates a stronger product, while decreasing chain length will yield a relatively weaker perfluorosurfactant (Fig. 1.6) (Krafft & Riess 2015).

The unique mix of properties make fluorosurfactants extremely versatile in the creation of consumer products and in manufacturing applications. The surfactant properties are utilized extensively in the creation of perfluoropolymers by way of emulsion polymerization (Krafft & Riess 2015). The surface activity of PFASs is also employed in fire-fighting foams to create a film able to suffocate fuel fires. The fluidic and thermal traits are exploited as additives in automotive and avionic hydraulic fluids to enhance stability and functionality at extreme temperatures. PFAS oil and water repellency characteristics are used as films on food packaging and paper products to prevent product destruction (Krafft & Riess 2015).

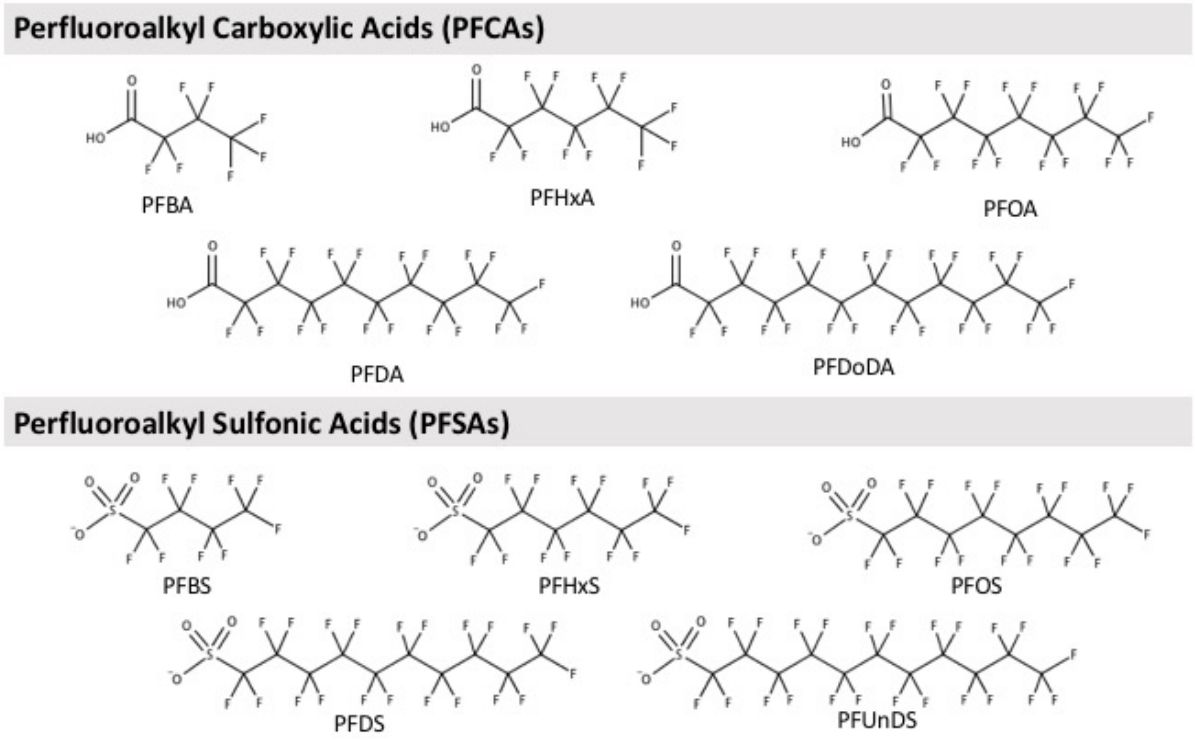

Figure 1.7: Different Structures Demonstrating Variation in PFAS Compounds. Abbreviations: perfluorobutanoic acid (PFBA); perfluorohexanoic acid (PFHxA); perfluorooctanoic acid (PFOA); perfluorodecanoic acid (PFDA); perfluorododecanoic acid (PFDoDA); perfluorobutane sulfonic acid (PFBS); perfluorohexane sulfonic acid (PFHxS); perfluorooctane sulfonic acid (PFOS); perfluorodecane sulfonic acid (PFDS); perfluoroundecane sulfonic acid (PFUnDS). Adapted from Lee and Choi, 2017.

1.2.2 PFAS Exposure

Production plant emission encompasses the largest source of environmental contamination and exposure with production waste contributing to air and water pollution to the surrounding areas (Ahrens & Bundschuh 2014). It is estimated that some 6900 tons of PFCAs have been emitted to the environment from direct sources since their creation in the 1950s up to 2006 (Prevedouros et al. 2006). Indirect contamination occurs largely from the breakdown of fluorotelomeres and fluoropolymers found in landfills and contributes a lesser amount to the total global emission of PFAS compounds, estimated at 350 tons from the 1950s to 2006 (Prevedouros et al. 2006). Environmental transport of

PFAS compounds is a major contributing factor to the dispersion of PFASs to rural areas. Flowing rivers, ocean currents, and air currents are all capable of carrying PFAS compounds large distances (Prevedouros et al. 2006). As a result, compounds have been detected in air, water, and soil samples in many different locations around the world including the arctic (Ahrens & Bundschuh 2014, Prevedouros et al. 2006).

Human exposure to PFAS compounds can occur from nearly all routes of exposure, however the primary route is ingestion (Fromme *et al.* 2007, Jain 2014). Contamination of surface water leads to direct contamination of both drinking water and water biota such as fish. PFAS contamination is also biomagnified through the food chain into other potential sources of contamination (Denys *et al.* 2014). As such, PFASs have been found in meat, eggs, milk, root vegetables, fruits, and processed snack foods (Jain 2014). Ingestion of contaminated drinking water is a further concern in areas with ill-equipped water treatment facilities (USEPA 2014). PFAS compounds have excellent bioavailability from oral exposure, resulting in nearly all of the ingested PFASs being found systemically (Cui *et al.* 2010, Jian *et al.* 2018).

Inhalation is the second most prominent route of exposure (Harris *et al.* 2017, Hinderliter *et al.* 2006). While PFAS compounds can be found in the atmosphere, atmospheric release is determined to be a small portion of total plant emissions (5%) (Prevedouros et al. 2006). Further, small amounts of PFASs released would quickly dissipate, therefore most direct inhalation exposure is found to be in those in close proximity to manufacturing plants or individuals working with products that may contain PFASs. PFAS inhalation can also occur, however, from dust found in the home and can contribute to overall PFAS exposure (Harris et al. 2017, Fraser *et al.* 2013). Dermal

exposure is the route least likely to cause significant contamination. Dermal exposure to PFAS compounds typically occurs from residual short-chain fluorosurfactants on fabrics or degradation of fluoropolymer coatings, however this exposure source appears to be quite minimal (Fasano *et al.* 2005).

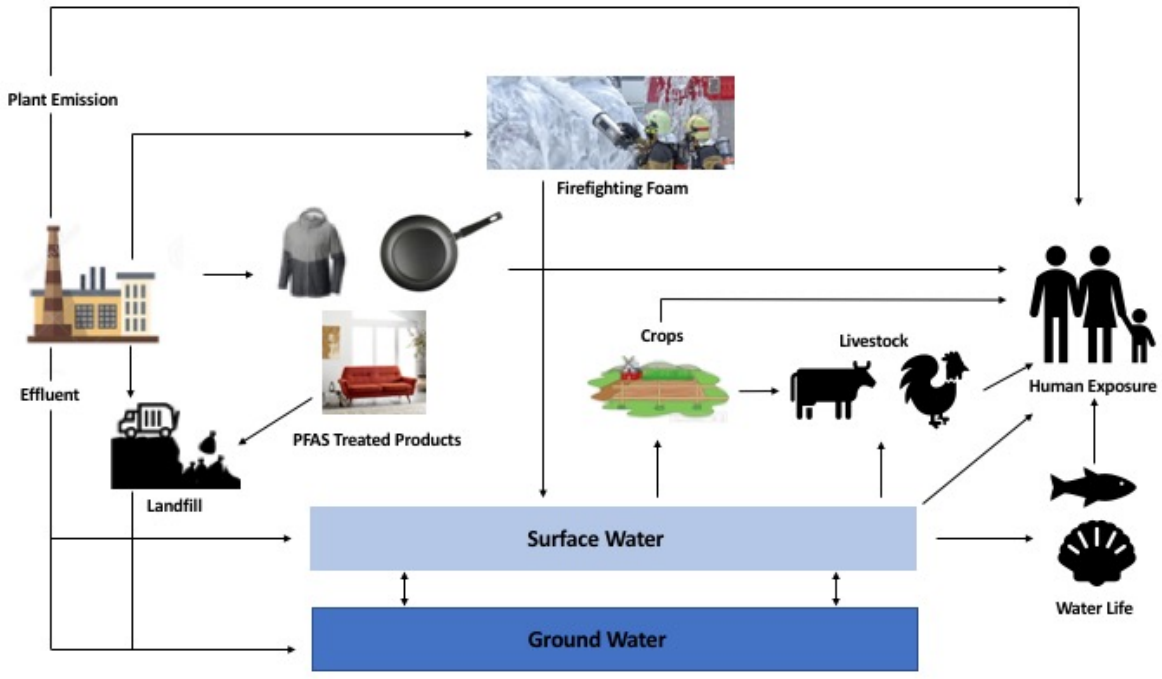

Figure 1.8: Demonstration of Human Exposure Routes from PFAS Sources. Adapted from Oliaei *et al.,* 2017.

1.2.3 PFAS Pharmacokinetics

Absorption: Membrane bound transport proteins and passive diffusion facilitate the absorption of PFAS compounds. The physicochemical properties of PFASs result in carrier-mediated uptake being more energetically favorable than passive diffusion. Specific transporter families have been identified to facilitate PFOA movement across

tissue membranes. Notably, the organic anion transporter (OAT), organic anion transporting polypeptide (OATP), and multidrug resistance-associated protein (MRP) families are known to assist in movement of PFOA across membranes of the gut, lung, and kidney (Klaassen & Aleksunes 2010, USEPA 2014). Currently, little is known about specific transporters assisting in the movement of PFOS across barrier membranes, however it is postulated that the same transporter families are involved in the movement of PFOS due to an increase in mRNA expression in tissues with PFOS present (USEPA 2016). *In vivo* experiments evaluating absorption of PFOA and PFOS demonstrate that both compounds are highly absorbed by the gut lumen after oral exposure, demonstrating 90% absorption within 48-hours of treatment (USEPA 2014, USEPA 2016, Chang *et al.* 2012, Cui et al. 2010). Little information is available regarding inhalation absorption of both PFOA and PFOS. However, one study of PFOA inhalation in rodents was identified to be proportional to the amount administered via nose inhalation after analyzing serum (Hinderliter et al. 2006). In another rodent study, PFOS was found to be absorbed after inhalation exposure, however full pharmacokinetic data was not included in the publication (USEPA 2016). Dermal absorption of PFOA is suggested to occur in rodent models (USEPA 2014). However, A later *in vitro* study conducted by measuring percutaneous absorption in rat and human skin demonstrated that absorption occurs much faster in rat skin than in human skin, raising skepticism to the importance of dermal absorption to overall PFOA exposure in humans (Fasano et al. 2005). Published reports investigating the dermal absorption of PFOS were not identified.

Distribution: After absorption, PFAS compounds are carried and distributed in systemic blood flow bound to serum proteins. Distribution of PFAS compounds in the

blood encompasses the largest fraction of PFAS distribution in the body due to the affinity of PFAS compounds for serum proteins. It is estimated that greater than 97% of PFAS compounds found in the body are located in serum (USEPA 2014, USEPA 2016). PFOA and PFOS are distributed in humans in blood serum, liver, bone, kidney, and lung (Perez et al. 2013, USEPA 2014, Olsen *et al.* 2003b). Small amounts of PFOA and PFOS have also been found in brain tissue and cerebrospinal fluid suggesting that PFOA and PFOS are able to cross the blood brain barrier (BBB) (Perez et al. 2013, USEPA 2014, USEPA 2016). However, the CSF:blood serum ratio for PFOS indicates that these compounds may not cross the BBB easily (Harada *et al.* 2007). PFOA and PFOS have also been found in umbilical cord blood and breast milk (Karrman *et al.* 2010, Apelberg *et al.* 2007, Volkel *et al.* 2008). This finding suggests that PFAS compounds may be transferred from mother to the developing child.

Metabolism: Due to the stability and inert properties of PFAS compounds, there has been no evidence to suggest PFAS compounds undergo metabolic processes within the body. This finding is further illustrated by the presence of only the anionic parent compound being found in urine, fecal, liver, and blood samples of animals dosed with PFAS compounds (Ylinen & Auriola 1990).

Excretion: Excretion and elimination of PFASs from the body can occur through routes of renal, hepatic, and biliary pathways. The main route of excretion appears to be from the kidneys through urine elimination. In Wistar rats administered a bolus I.P. injection of PFOA, fecal elimination accounted for less than 5% of administered dose (Kudo *et al.* 2001). However, in cynomolgus monkeys administered a bolus I.V. injection of PFOA, renal excretion accounted for the majority of the administered dose (Butenhoff

et al. 2004). This is thought to be due to the presence of OATs, OATPs, and MRPs expressed in the kidney (Worley & Fisher 2015). Hepatic and biliary excretion accounts for a much smaller proportion of elimination than renal, possibly due to the lack of PFAS transporters and extent of enterohepatic circulation (Kudo *et al.* 2007).

Serum half-lives have been reported and estimated through various *in vitro*, *in vivo*, and pharmacokinetic modeling studies. In humans, a study of biological half-lives was modeled from samples taken from cohort of retired PFAS manufacturing plant workers. It was reported that PFOA and PFOS had biological half-lives of 3.8 and 4.5 years, respectively (Olsen *et al.* 2007). An *in vivo* study of human subjects in a community with contaminated ground water was also conducted to determine the biological half-life of PFOA in subjects not exposed to high amounts of PFOA on a regular basis. The estimated half-life in this community was reported to be 2.3 years (Bartell *et al.* 2010). The extensive biological half-lives of PFAS compounds allow for bioaccumulation to take place, further exacerbating the potential impacts of PFASs on the body. This occurrence allows for even low exposure levels to build up over time to reach levels that may be detrimental to human health.

Wide ranges of PFASs have been reported in human serum from both occupationally exposed cohorts as well as cohorts of community members near manufacturing plants and cross sections of geographical land area. An extensive analysis of serum levels of PFOA in a cross section of the United States population, NHANES, reported a mean serum PFOA concentration to be 70.9 ppb (0.17 uM) (Winquist & Steenland 2014). A study analyzing PFOA serum concentrations in occupationally exposed workers reported a concentration of 2,210 ppb (5.34 uM) (Olsen & Zobel 2007).

The ranges of PFOA in each cohort was considerable with some individuals in the NHANES study having non-detectable serum concentrations, while others had levels above the mean for occupationally exposed subjects. The mean from various studies using similar cohorts displays a wide range of serum concentrations, making it difficult to accurately identify serum concentrations for each population (Coperchini et al. 2017).

1.2.4 Impact of PFASs on the THs

In an epidemiological study comprised of 506 occupationally exposed workers, no association with changes in total T_4 (TT_4) or TSH was seen with PFOA exposure, however there was a negative correlation between increasing PFOA serum levels, free T_4 (FT_4) and total T_3 (TT_3) levels (Olsen & Zobel 2007). This finding is consistent with *in vivo* studies that have demonstrated reductions in serum TH concentrations with PFAS exposure. Sprague-Dawley rats administered PFOA (20 mg/kg) and PFOS (10 mg/kg) by oral gavage over a 5 day period reported a significant decrease in TT_4, FT_4, and TT_3 for both compounds as early as the first day of exposure (Martin *et al.* 2007). An additional study with Sprague-Dawley rats administered a bolus dose of PFOS (15 mg/kg) by oral gavage demonstrated a transient increase in FT_4 with a significant decrease in TT_4 and TT_3. The same study also reported an increase in excretion of THs, suggesting the decrease serum THs was a result of a change in pharmacokinetics (Chang et al. 2008).

Further evidence of PFASs affecting serum THs is given by association of PFAS compounds and disease states. A cross sectional analysis of the U.S. population using 1,181 subjects reported an increased incidence of women with subclinical hypothyroidism in the highest exposure quartile for PFOA and PFOS reporting odds ratios for disease

development at 7.41 and 3.03, respectively. This represents the odds of an association between being exposed to PFAS compounds and presenting subclinical hypothyroidism. The same study concluded that men in the same exposure quartile for PFOS had increased incidences of subclinical hypothyroidism, with an odds ratio of 1.98 (Melzer *et al.* 2010). A similar cross-sectional study of the U.S. population set out to determine a correlation of PFOA and PFOS serum concentrations and thyroid disease in 3,974 subjects. The study reported an association of incidence of thyroid disease (unspecified) in the highest quartile exposure of PFOA in women with an odds ratio of 2.24. The same study reported an association of incidence of thyroid disease in the highest quartile exposure range of PFOS in men (Winquist & Steenland 2014).

PFASs also pose a serious threat to mothers and children in developmental stages of pregnancy, birth, and infancy. In a study of 285 pregnant women and neonates in the third trimester of pregnancy, maternal PFAS levels were associated with maternal serum and cord blood levels of TH. The study found that pregnant women with the highest exposure levels of three PFAS compounds, perfluoronanoic acid (PFNA), perfluoroundecanoic acid (PFUnDA(C=11)), and perfluorododecanoic acid (PFDoDA(C=12)), demonstrated a significant negative correlation with TT_4 and FT_4 in serum. The same compounds were found to have a significant negative correlation with TT_3 and TT_4 in cord blood, suggesting that these three PFAS compounds are able to alter thyroid hormones reaching the fetus (Wang *et al.* 2014).

An *in vivo* study evaluating neonatal mortality and pharmacokinetics in maternally exposed Sprague-Dawley rats, mothers were administered PFOS (0.4-2.0 mg/kg/day) via oral gavage for 42 days prior to mating. A significant decrease in TT_4 was demonstrated

at all exposure levels, and a significant decrease in TT_3 was seen with exposure groups above 1.0 mg/kg/day. The same study evaluated the TH levels of pups born to mothers exposed to PFOS and reported a significant decrease in TT_4 at all exposure levels and decreased FT_4 at exposure levels above 0.8 mg/kg/day. This study also reported a significant decrease in pup viability at the highest dose exposure levels (1.6 and 2.0 mg/kg/day), however, the change in serum TH was not found to be an associated cause of mortality (Luebker *et al.* 2005).

The exact mechanistic understanding for decreases in circulating TH levels is still not completely understood. However, an apparent change in TH pharmacokinetics is consistently noted during PFAS exposure (Chang et al. 2008, Yu *et al.* 2009). A variety of hypotheses have been constructed and tested through *in vitro* and *in vivo* studies in an effort to better understand the impact on PFASs on circulating TH levels. The conclusions drawn suggested that 1) PFAS compounds are able to up-regulate the expression of metabolic enzymes to enhance the rate of TH conversion and excretion, and 2) competitive binding between TH and PFASs in serum lead to changes in the free (bioavailable) fraction of TH leading to changes in circulating TH levels (Coperchini et al. 2017). The ability for PFASs to up-regulate metabolic enzymes is reportedly due to PFASs acting as antagonist for the transcription factor PPARα (Yu et al. 2009). The principle metabolic enzymes up-regulated by PFAS exposure are thought to be DIO2, which is involved in conversion of T_4 to T_3, and UDP-glucuronosyltransferase (UGT), which is involved in facilitating TH excretion.

1.2.4.1 PFAS Interaction with TH Uptake

It is well documented that both PFAS and TH compounds utilize carrier-mediated transport mechanisms to traverse the plasma membrane to enter the cell. Interestingly, PFAS compounds and THs may even utilize the same transport carriers to enter the cell highlighting a potential interaction that may occur between PFASs and THs at the site of transport. The table (table 1.4) below demonstrates the overlapping transporters that have been previously characterized for the uptake of PFOA, PFOS, and T_4. Overlapping transporter characterizations could suggest that PFAS compounds are able to inhibit the transport of T_4 into the cell, leading to a decrease in intracellular TH stores needed for proper function. This could serve as a potential mechanism by which PFASs could impact systemic THs.

Table 1.4: Overlapping Transport of PFASs and THs. The following table demonstrates transporters characterized for uptake of T4, PFOA, and PFOS as well as tissues expression. (h) prefix denotes human protein, (r) prefix denotes rodent protein.

Transporter	T_4	PFOA	PFOS	Location	References
rOatp1a1	x	x	x	Liver, kidney, choroid plexus	Visser et al. 2011, Bergwerk et al. 1996, Angeletti et al. 1997, Han et al. 2012
hOATP1A2	x			Kidney, Liver, intestines, BBB	Hagenbuch & Meier 2004, Visser et al. 2011
hOATP1B1	x	x	x	Liver	Hagenbuch & Meier 2004, Visser et al. 2011, Zhao et al. 2017
rOatp1b2	x		x	Liver	Visser et al. 2011, Cattori et al. 2000, Zhao et al. 2017
hOATP1B3	x	x	x	Liver	Hagenbuch & Meier 2004, Visser et al. 2011, Zhao et al. 2017
hOATP2B1	x	x	x	Liver, Intestines, placenta, spleen, lung, kidney, heart, ovary, brain	Hagenbuch & Meier 2004, Visser et al. 2011, Zhao et al. 2017
hOATP1C1	x	x	x	BBB, testis	Hagebuch & Meier 2004, Visser et al. 2011
rOatp4a1	x			Ubiquitous	Visser et al. 2011, St-Pierre et al. 2004
hOATP4C1	x			kidney	Visser et al. 2011, Sato et al. 2017
hOAT1		x		Kidney, choroid plexus	Nigam et al. 2015, Han et al. 2012
hOAT2		x		Kidney, liver	Nigam et al. 2015, Han et al. 2012
hOAT3		x		Kidney, eye, brain	Nigam et al. 2015, Han et al. 2012
hMCT8	x			Neurons, astrocytes, liver, thyroid, kidney, heart, testes	Visser et al. 2011, Halestrap & Wilson 2012, Friesema et al. 2003
hMCT10	x			Microglia, neurons	Visser et al. 2011, Halestrap & Wilson 2012
hLAT1	x			Neurons, astrocytes, microglia, BBB	Visser et al. 2011, del Amo et al. 2008
hLAT2	x			Neurons, oligodendrocytes, BBB	Visser et al. 2011, del Amo et al. 2008
hNTCP	x		x	Liver	Visser et al. 2011, Sun et al. 2001, Zhao et al. 2015

1.2.4.2 PFAS Interaction with TH Serum Proteins

The impact of competitive binding of PFAS and TH in serum is thought to be a principle mechanism of PFAS mediated changes in serum TH levels. Due to the extensive binding of THs in serum (>99%), only a small amount of TH remains free and available for cellular uptake and activity (Mondal et al. 2016, Refetoff 2000). Therefore, even a slight change in the free fraction of THs caused by PFAS could dramatically increase the free fraction of THs in serum. Multiple *in vitro* studies have been conducted to confirm the ability of PFAS compounds to disrupt TH binding to their three serum proteins. An analysis of inhibitory binding impact of 24 different PFAS compounds on TH binding to TTR found

11 PFAS compounds able to inhibit >50% of T4 binding. Of the compounds tested, those displaying inhibitory impact on TTR were between 5-10 carbons in length, demonstrating a range of PFAS size optimal for T_4 binding inhibition to the TTR protein. Of note, PFOA and PFOS both displayed IC_{50} values less than 10 μM, which would be within relevant human exposure levels (Weiss *et al.* 2009). A similar study was conducted to evaluate PFAS binding impact on TTR and TBG. This study showed results consistent for PFAS binding to TTR and demonstrated reasoning for TTR binding specificity for PFAS based on computational molecular docking, demonstrating the TTR binding pocket to have an optimal range of PFAS sizes for linear PFAS compounds between 5-10 carbons. Again, PFOA and PFOS demonstrated the most potent inhibitory affinity on TTR (Ren *et al.* 2016). The competitive impacts of PFAS compounds did not seem to translate to TBG. No significant binding inhibition of T_4 was detected for any of the PFAS chemicals tested, demonstrating the impact of PFAS compounds to be more specific to TTR.

Studies investigating competitive displacement of TH binding to albumin with PFASs have not been published to date. There is a body of evidence demonstrating that PFASs and THs both have an ability to bind to albumin, however the existence of multiple binding sites may present the ability of PFAS and TH to coexist on the same albumin molecule. Further studies demonstrating the ability, or lack thereof, for PFAS to competitively displace THs from albumin are needed in order to gain a full understanding of serum TH displacement.

1.2.4.2 Impact of Displacement on TH Uptake

While it is well established that PFASs are able to alter the free fraction of TH in serum, it is not understood how this effect could impact TH uptake. However, there is sufficient evidence to suggest that the extent of protein binding can impact substrate uptake. *In vitro* and *in vivo* analysis of how protein binding impacts uptake of the compound deltamethrin demonstrates that an increase in serum protein binding causes a decrease in uptake at the blood brain barrier (Amaraneni *et al.* 2017, Amaraneni *et al.* 2016). In other words, a change in the unbound fraction of deltamethrin caused a change in uptake. Deltamethrin and thyroxine are both highly lipophilic compounds that are extensively protein bound in serum. Therefore, it is possible that a similar phenomenon could take place as PFASs are able to alter the unbound fraction of THs. Altering the amount of uptake could result in an increase in TH excretion, particularly in organs such as the liver and kidney.

1.3 Rationale and Aims

PFAS compounds are able to decrease serum concentrations of THs *in vivo*. However, the mechanisms involved for this action are not completely understood. It is proposed that PFASs are able to increase expression of TH deactivating metabolic enzymes, such as UGTs, to increase TH excretion. This is in conjunction with data demonstrating that PFASs are able to displace TH from serum binding proteins, altering the unbound hormone fraction. What has not yet been evaluated is the impact of PFASs on TH uptake across the barrier that separates the aforementioned increased free fraction

and increased enzyme expression. This rate limiting step may be a critical link that provides further insight to understand how PFASs are able to decrease systemic THs.

Previous work in our laboratory has demonstrated that uptake of highly lipophilic and extensively protein bound compounds depends upon the extent of protein binding. Therefore, a change in the unbound fraction can have a drastic impact on the rate of substrate uptake. Substrate uptake has been coined as a “phase 0” step of metabolism due to its role as a rate limiting step of intracellular metabolism (Doring & Petzinger 2014). Given the ability of PFASs to alter the unbound fraction of THs in serum, it would follow that the change in unbound fraction could present a dramatic increase in the “phase 0 transport” step of metabolism, leading to excess intracellular THs available for metabolic deactivation and excretion. Conversely, the overlap in transporters characterized for PFAS and TH uptake could suggest PFASs may even present the ability to inhibit T_4 uptake. The purpose of this study is to identify how two widely distributed PFAS compounds, PFOA and PFOS, are able to impact the rate of TH uptake. We hypothesize that PFOA and PFOS will be able to alter the rate of T_4 uptake. We will investigate the potential T_4 displacement caused by PFOA and PFOS causing an increased rate of T_4 uptake (Fig. 1.9). We will also investigate the ability of PFASs to inhibit T_4 carrier mediated uptake.

Given their abundance of TH transport proteins, rodent hepatocytes will be used to evaluate PFAS effects on T_4 uptake. We will also evaluate T_4 uptake in a specific transporter using an overexpression model to further assess the impact of carrier-mediated transport in hepatic uptake. Understanding the mechanisms involved in PFAS mediated effects on THs could provide useful insight in risk assessment of PFAS

compounds in human health, as well as contributing to an understanding of how xenobiotics are able to impact the HPT axis.

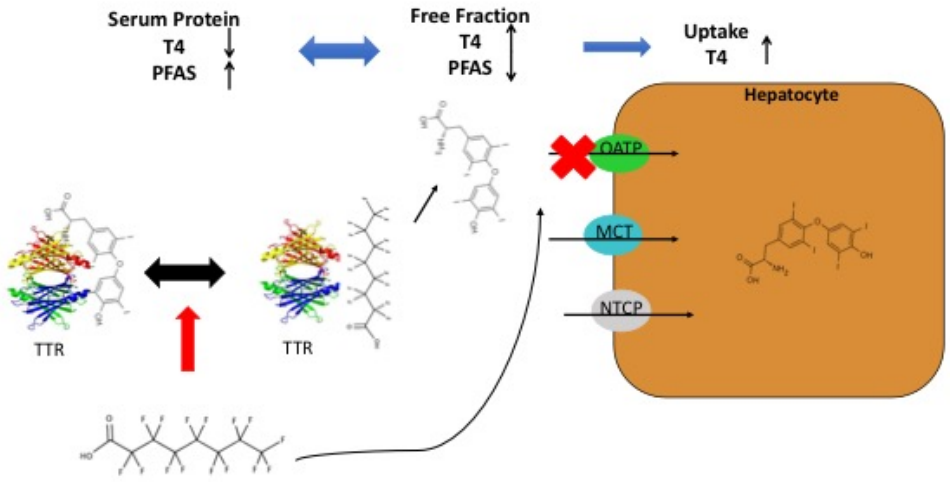

Figure 1.9: Pictorial Demonstration of Hypothesis. Figure depicts hypothesis and research questions.

CHAPTER 2

The Impact of Perfluoroalkyl Substances on Hepatic T_4 Uptake

2.1 Introduction:

Previous research investigating PFAS effects on THs consistently implicate changes in free TH fraction and an increase in TH metabolism as a cause for decreases in systemic THs *in vivo* (Weiss et al. 2009, Ren et al. 2016, Yu et al. 2009). However, there is a lack of evidence demonstrating how PFAS compounds impact the uptake of THs into the cell. Understanding the effects of PFASs on TH uptake may be critical in developing a mechanistic understanding of the reduction in systemic TH concentrations (Hennemann et al. 2001). To demonstrate the impact of PFASs on TH uptake, isolated primary hepatocytes will be used to evaluate T_4 uptake in the presence of PFAS compounds and the serum protein TTR.

Isolated hepatocytes have been utilized extensively in evaluation of substrate uptake due to their functional expression of many transporters and ability to predict *in vivo* intrinsic clearance (Hallifax & Houston 2006). While cryopreserved human hepatocytes are available, their cost and amount of inter-donor variability are prohibitive characteristics (Menochet *et al.* 2012a, Menochet *et al.* 2012b). Conversely, cryopreserved rat hepatocytes present a cheaper alternative while still providing a basis for prediction of human hepatic disposition (Menochet et al. 2012a). Furthermore, experimental lots of cryopreserved rat hepatocytes offer a larger population of donors and less lot to lot variability. TH uptake has been previously characterized in isolated rat hepatocytes

(Hennemann et al. 2001). However, there are conflicting reports regarding the presence of carrier-mediated transport activity (Rao & Rao 1983, Hennemann et al. 2001). Initial analysis will require confirmation of T_4 carrier-mediated transport in order to evaluate the impact of PFASs on T_4 uptake.

The impact of PFAS compounds on the free TH fraction has been determined to be principally driven by an interaction with transthyretin (TTR) (Weiss et al. 2009, Ren et al. 2016). To date, PFAS compounds have not been found to impact TH binding to TBG or SA. As such, investigation of PFAS impact on uptake via free T_4 alterations will be conducted with TTR. Interestingly, carrier-mediated transport of PFAS compounds has been characterized in isolated rat hepatocytes and in specific transporters preferentially expressed in hepatocytes, such as OATPs (Zhao *et al.* 2017, Han *et al.* 2008). Therefore, identification of T_4 transport inhibition by PFASs will be necessary to ensure this does not confound how changes in the free T_4 fraction alter uptake.

Analysis of T_4 uptake has classically been performed with isotopically labeled THs. However, the use of radiolabeled substrates possesses limitations such as an inability to distinguish a parent compound from metabolite, cost of radioligand production, procurement of radioactive waste, and health concerns (Schmitt *et al.* 2014, Jayarama-Naidu *et al.* 2015). Utilizing analysis by liquid chromatography and tandem mass spectrometry (LC-MS/MS) provides advantageous properties that limit error and provide a safer working environment (Jayarama-Naidu et al. 2015). As such, analysis of T_4 uptake will be conducted with LC-MS/MS techniques to increase precision of T_4 detection and provide monitoring of T_3 production.

Two widely dispersed PFAS compounds, PFOA and PFOS, will be evaluated for their impact on T_4 uptake due to their prevalence in human exposure (Winquist & Steenland 2014, Frisbee et al. 2009, Olsen *et al.* 2003a). It is hypothesized that PFOA and PFOS will cause an increase in carrier-mediated T_4 transport as a result of changes in the free T_4 fraction. The first aim for this project will be to identify the ability of PFOA to inhibit T_4 uptake. Our second aim will be to evaluate the impact of PFOA and PFOS on the T_4 uptake in the presence of TTR. Identification of PFAS effects on T_4 uptake will provide further mechanistic insight into how PFASs decrease systemic THs *in vivo*.

2.2 Methods:

2.2.1 Chemicals and Reagents

L-Thyroxine (T_4; (2S)-2-amino-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5diiodo-phenyo] propanoic acid), (T_3; (2S)-2-amino-3-[4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl] propanoic acid), cyclosporine A (CsA), PFOS, silicone oil, and mineral oil were purchased from Sigma Aldrich (St. Louis, MO). PFOA was purchased from Oakwood chemicals (Estill, SC). $^{13}C_6$-labeled T4 ($^{13}C_6$-T_4) was purchased as an internal standard from Cambridge Isotope Laboratories (Tewksbury, MA). William's media, hepatocyte thaw media (Invitrogen; CM7500) and hepatocyte cell maintenance supplement (CM4000) were purchased from Invitrogen (Waltham, MA). The serum protein transthyretin (TTR) derived from human plasma was purchased from Athens Research & Technologies (Athens, GA). Evolute Express (CX, 10 mg) 96-well solid-phase extraction (SPE) plates were purchased from Biotage (Charlotte, NC). Microcon® centrifugal filter devices with Ultracel YM-10 membrane (10,000 Da nominal molecular

weight limit) were purchased from Millipore (Bedford, MA). All solvents and acids used in extraction and elution were of Optima-Grade performance and purchased from Thermo Fisher (Pittsburgh, PA).

2.2.2 Cryopreserved Hepatocytes

Cryopreserved rat hepatocytes were purchased from Bioreclamation IVT (Baltimore, MD). Hepatocytes are derived from livers of pooled male Sprague-Dawley rats containing 4 to 6 donors depending on lot. Lots purchased and used in experimentation are CZX and SNG.

2.2.3 T_4 Hepatocyte Uptake Assay

With Transthyretin

Cryopreserved rat hepatocytes were thawed in hepatocyte thawing media (Invitrogen; CM7500) and brought to a concentration of $2.0x10^6$ cells/mL in running buffer (William's media supplemented with hepatocyte cell maintenance supplement) at 37°C and equilibrated for 10 mins. For assays performed at 4°C, cells were equilibrated on ice for an additional 15 mins following previous 10-minute equilibration at 37°C. Following equilibration, aliquots of $0.3x10^6$ cells were assayed individually with T_4 (1 µM) in the presence of CsA (0.1-10 µM), PFOA (0.1-10 µM), PFOS (10 µM), or vehicle control (DMSO) and maintained at 37°C or 4°C. mixtures of T_4 and treatment were mixed with TTR (0-125 µg/mL) and set at 37°C for approximately 30 min to allow for equilibration with serum binding proteins. For assays at 4°C, TTR and chemical mixtures of T_4 with PFOA, PFOS, OA, CSA, or DMSO were placed on ice for an additional 15 min following

30 min equilibration at 37°C. The assay was initiated with addition of cells to the substrate mixture and commenced at 15, 30, 60, and 90 sec (37°C) by placing aliquots of $5.0x10^4$ cells on oil-filtration tubes and commencing centrifugation at 18,440 x g for 30 sec immediately (Hettich MIKRO220). Oil-filtration tubes contained a layer of silicone:mineral oil (5:1) atop of a layer of 0.5 M cesium chloride. Assays conducted at 4°C were commenced at 30, 60, 90, and 120 sec following the same process. These steps were repeated to gain 3 individual uptake rates (n = 3) for each treatment group. After experiments were completed, oil-filtration tubes were placed in a -80°C freezer for at least 1 hour. After samples were thoroughly frozen, oil-filtration tubes were cut through the oil layer. The bottom layer containing the cell pellet was collected for extraction and analysis.

Without Transthyretin

For uptake assays conducted in the absence of TTR, assay procedures were the same as previously described. However, only half of the cell concentration (collecting final aliquots of $2.5x10^4$) was used due to sufficient T_4 analytical response in the absence of serum binding proteins.

2.2.4 T_4 Quantification

T_4 extraction

Previously collected uptake assay samples were thawed at room temperature and lysed with the addition of acetonitrile:water mixture (80:20 %) containing 4% formic acid (FA). The samples were vortexed and placed on a mini orbital shaker (Versa-ORB) at 150 rpm for 10 min at room temperature, then centrifuged at 18840xg (Hettich

MIKRO220) for 10 min to pellet any residual oil or cell debris. An aliquot of supernatant was removed and placed into a separate microcentrifuge tube with internal standard ($^{13}C_6$-T_4,100 ng/mL, 0.1M NaOH), and diluted with water containing 4% FA for solid phase extraction.

T_4 solid phase extraction

Samples were processed through solid phase extraction (SPE) using a 96-well cation exchange plate (Evolute Express CX, 10 mg, 1mL, Biotage, Charlotte, North Carolina) and a vacuum filtration manifold (Multiscreen HTS, Millipore). The SPE plate was conditioned with methanol followed by water containing 2% FA. Sample extracts were then loaded onto the SPE plate and vacuumed to dryness. Each well was then washed with water containing 2% FA followed by methanol. T_4 was then eluted from the SPE sorbent using a triplicate rinse with a mixture of methanol:acetonitrile (50:50%) containing 5% NH_4OH into a 96 well plate. Samples were then evaporated to dryness using Microvap nitrogen dryer (Organomation, Berlin, MA), and reconstituted in LC-MS/MS mobile phase (60:40% methanol:water) for LC-MS/MS analysis.

LC-MS/MS analysis

The SPE reconstituted samples were analyzed for T_3 and T_4 using an Agilent 1200 Ultra-performance liquid chromatograph (UPLC) coupled to a 6420 triple quad mass spectrometer (Agilent, Santa Clara, CA). Injections (5 uL) at a 1.0 ml/min flow rate were made onto an Agilent Zorbax XDB-$_{C18}$ column (4.6 mm x 50 mm, 1.8 um particle diameter; Santa Clara, CA) maintained at 40°C. Gradient elution with methanol (solvent A) and

water (solvent B) with 0.2% FA was applied under the following conditions: 60% A for 0.5 min, followed by a linear gradient to 70% A at 3.0 min, increasing to 100% A at 3.2 min, and held for a 6.5 min stop time. The column was then allowed to re-equilibrate under the original conditions for a 3 min post-time. MS/MS detection was conducted using ESI+ in multiple reaction mode under the following conditions: T_4 quantifying transition ion *m/z* 777.7-731.5, with qualifying ion transitions *m/z* 777.7-633.5 (collision energies 25V), with the fragmenting voltage set to 160 V and the cell accelerator at 7 V. T_3 quantifying transition ion *m/z* 651.8-605.9, with qualifying ion transition *m/z* 651.8-478.7 (collision energies 30 V and 35 V, respectively), with the fragmenting voltage set at 120 V and the cell accelerator at 7 V. The internal standard, L-Thyroxine ($^{13}C_6$ -T_4) was quantified based on the transition ion *m/z* 738.8-737.8 with the fragmenting voltage, collision energy, and cell accelerator set to 160 V, 25 V, and 7 V, respectively (Wang and Stapleton, 2010). ESI source parameters were applied according to the following: source gas temperature 350°C, gas flow 12 L/min, nebulizer 55 psi, capillary 4000 V. T_3 and T_4 standard curves (0.1–125 ng/mL) were prepared in (60:40, methanol:water) using L-Thyroxine ($^{13}C_6$ -T_4) as an internal standard and verified during analysis with a check standard every 12 samples, followed by a blank sample for carryover assessment. Data processing was performed using Agilent MassHunter software (version B.04.01) and sample concentrations were determined using an internal-standard response factor.

2.2.5 Determination of T4 Free Fraction

T_4 (1 µM) and TTR (62.5 µg/mL) were prepared in running buffer with PFOA (0.1-10 uM), PFOS (10 µM), Octanoic acid (OA) (10 µM), or vehicle control (DMSO). This

assay mixture was placed at 37°C for approximately 30 min to allow for equilibration. Assays conducted at 4°C were placed on ice for an additional 15 min to mimic uptake assay conditions. Aliquots of assay mixtures were placed in ultrafiltration devices containing a 10 kD filtration membrane (Microcon ultracel YM-10, Millipore) and centrifuged at 18440xg for 5 min (Hettich MIKRO220). The resulting filtrate was collected and prepared for LC-MS/MS analysis following T_4 quantification procedures.

2.2.6 Data preparation and Statistical analysis

Rates of total uptake (37°C) and passive diffusion (4°C) were derived from the slope of linear regression through timepoints collected from hepatocyte uptake assays. Quality criteria for each assay followed that the r^2 of linear regression must be >0.7. Differences in uptake rates between treatment (CsA, PFOA, PFOS, OA) and control groups (DMSO) at 37°C and 4°C were determined using one-way or two-way ANOVA analysis with Tukey's post hoc test of the derived rate of uptake ± SD using Graphpad prism 7 software.

Estimation of the Michaelis-Menten constant (K_m) and maximal transport velocity (V_{max}) were conducted by comparing the rates of T_4 uptake to the concentration of T_4 used in analysis. Rates of carrier mediated uptake (37-4°C) were calculated by subtracting the rate of passive diffusion (4°C) from the rate of total uptake (37°C) at each T_4 concentration. Graphing and kinetic analysis were performed by applying a one-site Michaelis-Menten equation with a non-saturable component using Graphpad prism 7 software.

$$V = \frac{V_{max}[S]}{K_m + [S]} + K_{ns}[S]$$

Where V is the total rate of uptake, V_{max} is the maximum uptake rate, K_m is the dissociation constant, [S] is the substrate concentration, and K_{ns} is the coefficient for nonspecific uptake by diffusion.

Differences in the control and treatment groups of free T_4 fraction assays were compared using one-way or two-way ANOVA analysis with Tukey's post hoc test for the mean±SD using Graphpad prism 7 software.

2.3 Results:

2.3.1 Kinetic Analysis of Hepatic T_4 Uptake

Total T_4 uptake velocities conducted at 37^{o}C demonstrated saturable enzyme kinetics with respect to increasing T_4 concentration (0.1-15 µM) (Fig.2.1). In contrast, T_4 uptake velocities at 4^{o}C displayed a non-saturable linear relationship to increasing T_4 concentration, representative of passive diffusion (Fig.2.1). Michaelis-Menten data analysis applied to total T_4 uptake versus concentration resulted in a V_{max} value of 5.78 pmoles/10^6 cells/sec and a K_m value of 9.08 µM. Subtraction of the passive diffusion component from total T_4 uptake (37^{o}C-4^{o}C) yields the uptake velocity of carrier-mediated transport. Michaelis-Menten data analysis applied to carrier-mediated T_4 transport versus concentration demonstrated a V_{max} value of 3.28 pmoles/10^6 cells/sec and a K_m value of 5.05 µM. further assays were conducted at 1.0 µM T_4 to ensure substrate concentrations are maintained below the determined K_m value.

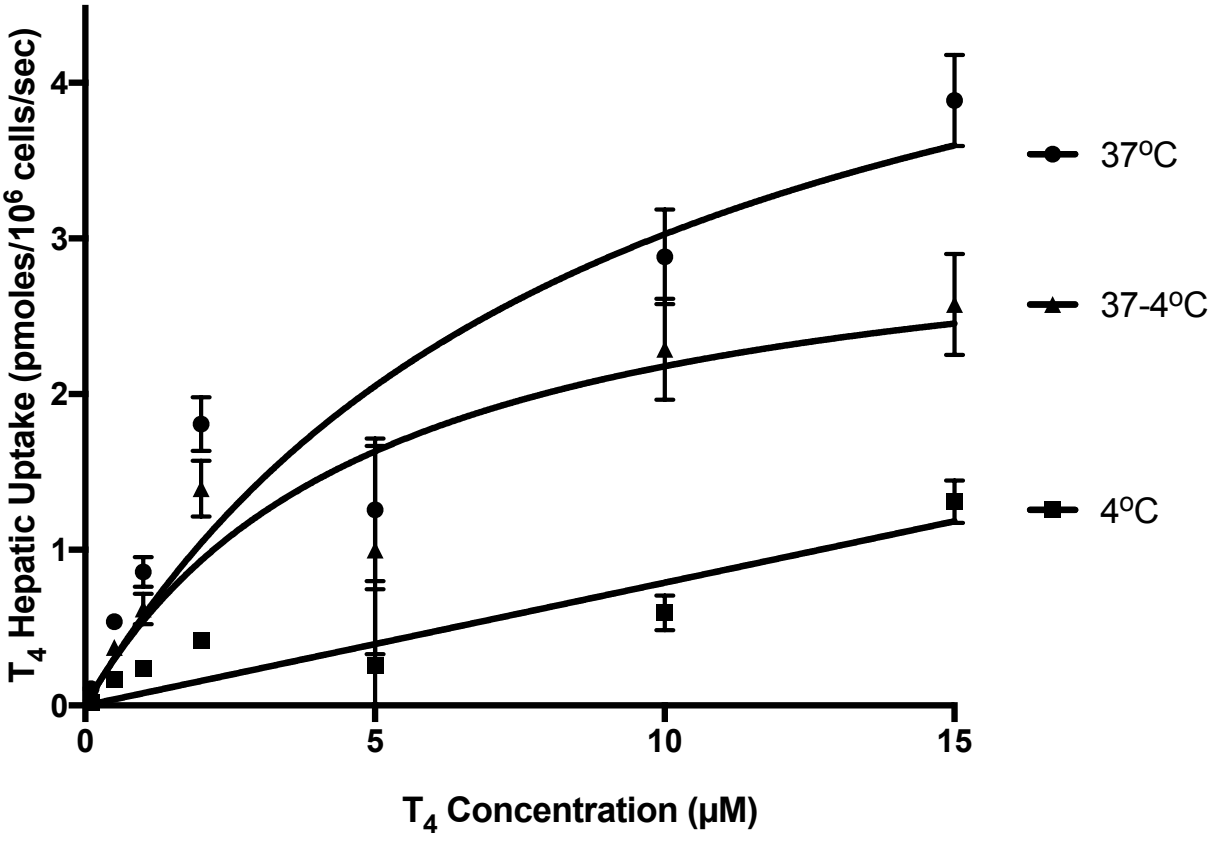

Figure 2.1: Kinetic Uptake of T_4: Substrate kinetic profiles of T_4 uptake in cryopreserved rat hepatocyte suspensions ● 37°C (carrier-mediated + passive diffusion), ▲ 37°C - 4°C (carrier mediated), and ■ 4°C (passive diffusion). Data represents slope of linear regression for each substrate concentration tested. Error bars represent ±SD with n = 3.

2.3.2 Inhibition of T_4 Uptake

Cyclosporine A (CsA), a broad transport inhibitor, was utilized to further demonstrate carrier-mediated T_4 transport (Fig. 2.2A) (Karlgren *et al.* 2012). A significant reduction of total T_4 uptake at 37^{o}C was determined at all concentrations of CsA evaluated when compared to the vehicle control (DMSO). A significant reduction in T_4 uptake at all concentrations also occurred at 4^{o}C compared to temperature control. However, the reduction was to a lesser extent at 4^{o}C than that of 37^{o}C. These finding suggests the presence and activity of carrier-mediated T_4 transport.

To determine if PFOA could inhibit T_4 transport, Hepatic T_4 uptake was evaluated with varying concentrations of PFOA in the absence of the serum binding protein TTR. In contrast to inhibition of T_4 demonstrated by CsA, PFOA demonstrated no significant change in total or passive T_4 uptake, indicating no impact on T_4 at the site of transport (Fig. 2.2B). However, a significant decrease in T_4 uptake at 4^{o}C was demonstrated at all concentration levels when compared to 37^{o}C, further suggesting the presence of a carrier-mediated transport component.

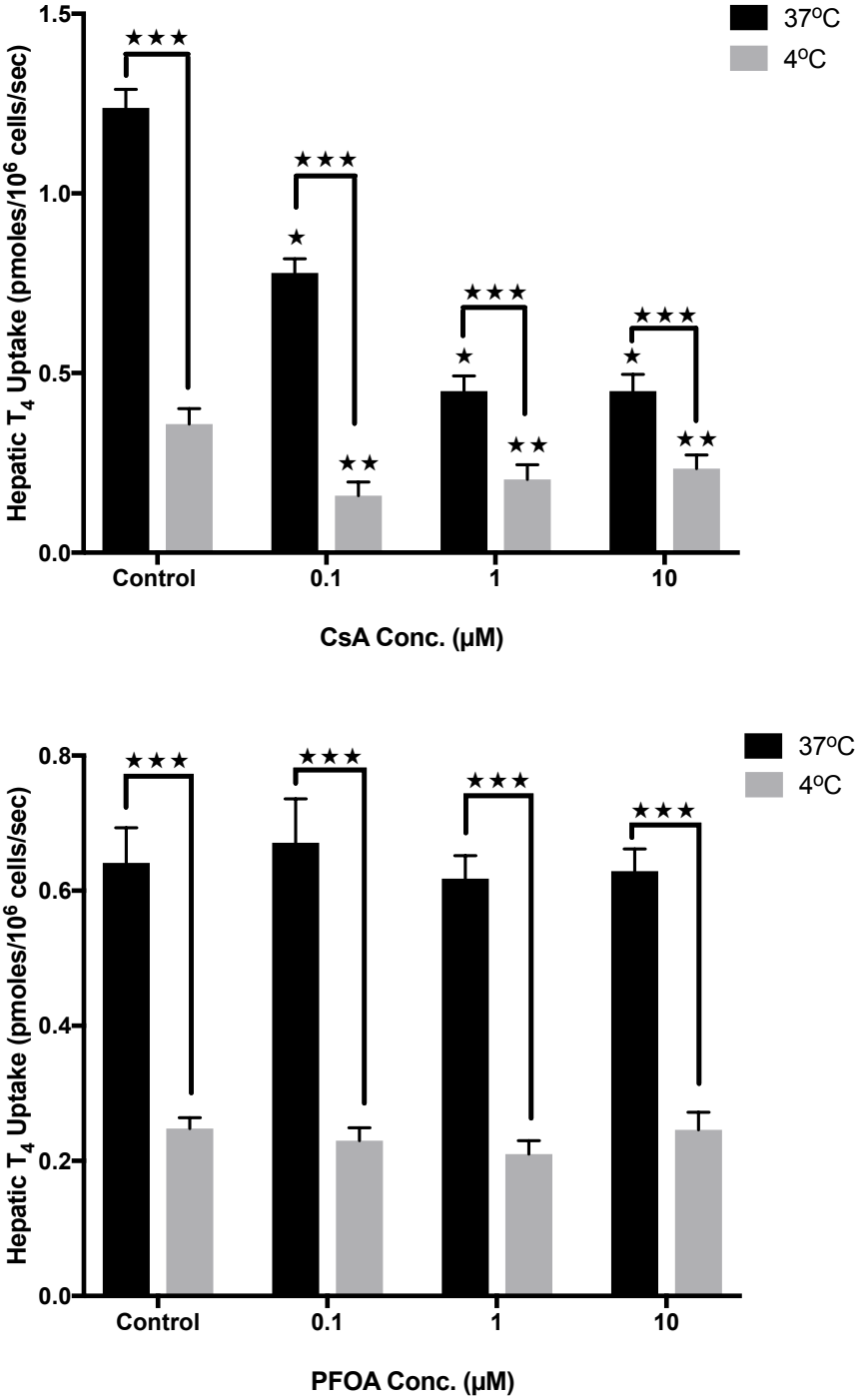

Figure 2.2: T_4 Hepatic Uptake. T_4 (1 μM) uptake was determined in hepatocytes treated with varying concentration of (A) cyclosporine A and (B) PFOA. Data represents uptake rate determined from the slope of linear regression (pmoles/10^6 cells/sec) ± SD from n=3 independent experiments. (★) represents a statistically significant difference ($p<0.05$) compared to 37ºC control, (★★) represents a statistically significant difference ($p<0.05$) compared to 4ºC control, (★★★) represents a statistically significant difference ($p<0.05$) between 37ºC and 4ºC. Data was analyzed with a two-way ANOVA with a Tukey's post-hoc test.

2.3.3 Effect of Protein Binding on T_4 Uptake

To evaluate the impact of the TH serum binding protein TTR on TH transport, T_4 hepatic uptake was evaluated in the presence of varying TTR concentrations. Results demonstrate a significant reduction in total hepatic uptake at 37^{o}C with increasing concentration of TTR, while no such effect is identified at 4^{o}C (Fig 2.3A). The free T_4 fraction was also evaluated under similar conditions to provide evidence that the rate of T_4 uptake is influenced by a change in the free TH fraction. An increase in TTR concentration also demonstrates significant reduction in the free T_4 fraction at both 37^{o}C and 4^{o}C (Fig. 2.3B). Correlation of the change in free T_4 fraction and change in hepatic T_4 uptake rate reveal a positive correlation of r = 0.99 at 37^{o}C (Fig. 2.3C) and r = 0.93 at 4^{o}C (Fig. 2.3D).

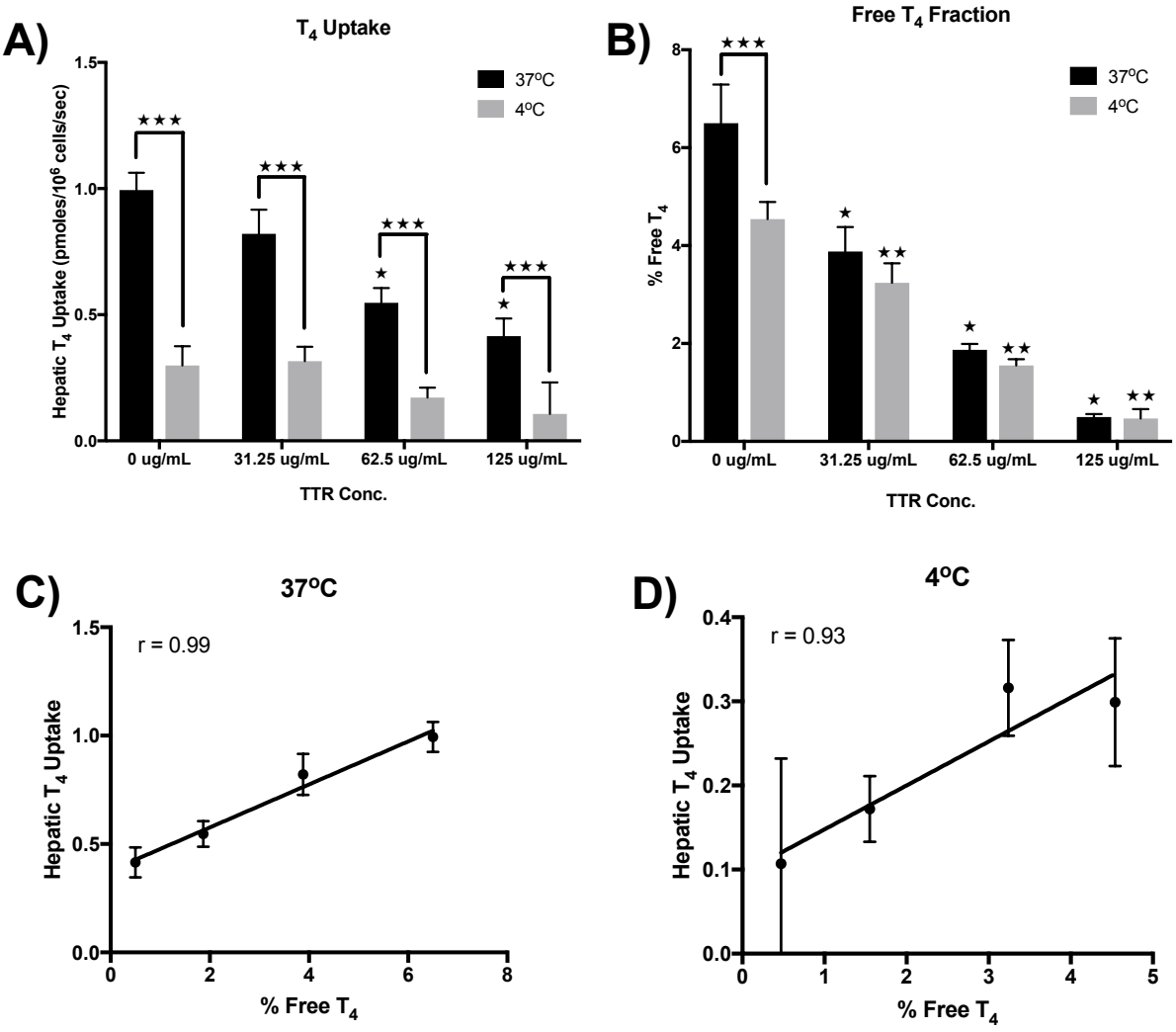

Figure 2.3: Impact of Serum Protein Binding on T_4 Uptake (A) Hepatic T4 uptake was evaluated with varying concentrations of the T4 serum binding protein, TTR. Data represents uptake rate determined from the slope of linear regression (pmoles/10^6 cells/sec) $\pm$ SD from n=3 independent experiments. (B) The free T4 fraction (%) was evaluated with varying concentrations of TTR. Data represents mean ± SD from n=3 independent experiments. (★) represents a statistically significant difference ($p<0.05$) compared to 37°C control, (★★) represents a statistically significant difference ($p<0.05$) compared to 4°C control, (★★★) represents a statistically significant difference ($p<0.05$) between 37°C and 4°C, (#) represents a statistically significant difference ($p<0.05$) between concentrations within a temperature range. Data was analyzed with a two-way ANOVA with a Tukey's post-hoc test. Correlation between the % free T4 and hepatic T4 uptake was determined at 37°C (C) and 4°C (D).

2.3.4 Effect of T_4 Displacement on Hepatic Uptake

To assess the impact of PFOA on T_4 uptake in the presence of TTR, PFOA concentrations were varied (0.1-10 μM) while maintaining a constant TTR concentration (62.5 μg/mL). Rates of T_4 uptake were increased significantly by the presence of 5, 8, and 10 μM concentrations of PFOA compared to the 37°C control. At 4°C only the 0.1 μM concentration of PFOA caused a significant increase in T_4 hepatic uptake compared to the temperature control. Significant increases in hepatic T_4 uptake are noted between the 1 and 5 μM concentrations and between the 8 and 10 μM concentrations of PFOA (Fig.2.4A). A significant difference in hepatic T_4 uptake was also noted between 37°C and 4°C at concentrations of 5, 8 and 10 μM PFOA (Fig. 2.4A).

The free T_4 fraction also demonstrated a significant increase at PFOA concentrations of 5, 8 and 10 μM compared to 37°C control (Fig. 2.4B). Interestingly, a significant increase in T_4 free fraction was also demonstrated at PFOA concentrations of 1, 8, and 10 μM compared to 4°C control (Fig. 2.4B). Correlation between increased free T_4 fraction and hepatic T_4 uptake rates demonstrate a strong positive correlation at 37°C (Fig. 2.4C), while no correlation was demonstrated at 4°C (Fig. 2.4D).

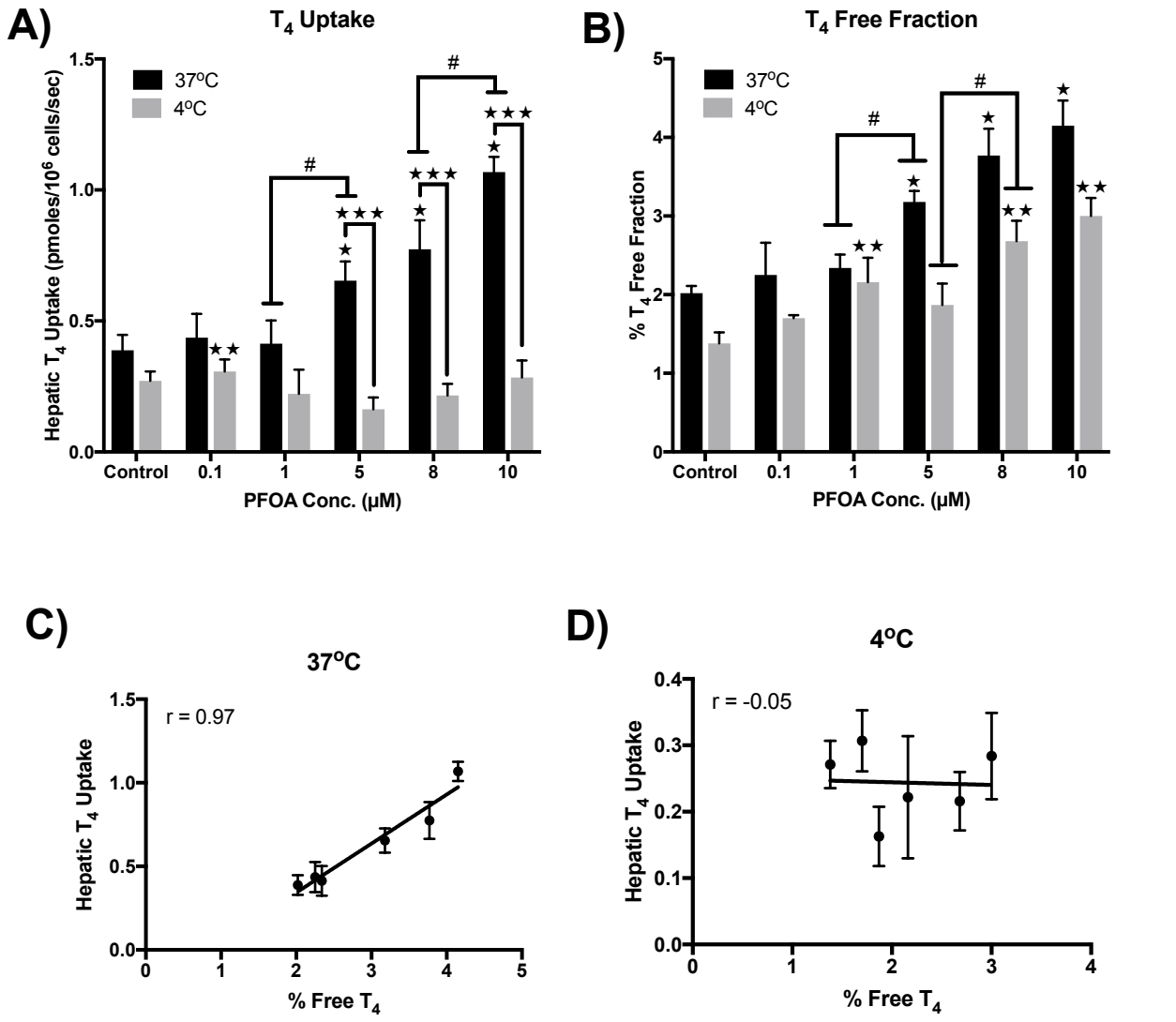

Figure 2.4: Impact of PFOA on T_4 Free Fraction and Hepatic Uptake. (A) Hepatic T_4 (1 μM) uptake was evaluated with varying concentrations of PFOA with TTR (62.5 μg/mL) present. Data represents uptake rate determined from the slope of linear regression (pmoles/10^6 cells/sec) ± SD from n = 3 independent experiments. (B) The free T_4 fraction (%) was evaluated with varying concentrations of PFOA with TTR (62.5 μg/mL) present. Data represents mean ±SD from n=3 independent experiments. (★) represents a statistically significant difference ($p<0.05$) compared to 37°C control, (★★) represents a statistically significant difference ($p<0.05$) compared to 4°C control, (★★★) represents a statistically significant difference ($p<0.05$) between 37°C and 4°C, (#) represents a statistically significant difference ($p<0.05$) between concentrations within a temperature range. Data was analyzed with a two-way ANOVA with a Tukey's post-hoc test. Correlation between the % free T_4 and hepatic T_4 uptake was determined at 37°C (C) and 4°C (D).

To further evaluate the impact of PFAS compounds on TTR, total hepatic T_4 uptake was evaluated in the presence of PFOS (10 μM). Similar to PFOA, PFOS demonstrated a significant increase in total hepatic T_4 uptake rate (Fig. 2.5A) and free T_4 fraction (Fig. 2.5B). Interestingly, A significant difference in T_4 uptake was also demonstrated between PFOA and PFOS for both hepatic T_4 uptake (Fig. 2.5A) and free T_4 fraction (Fig. 2.5B). suggesting PFOA to have a greater effect on T_4 displacement and uptake than PFOS. The naturally occurring fatty acid, octanoic acid, was also evaluated as an un-fluorinated negative control to PFOA. OA did not demonstrate a significant change in T_4 hepatic uptake (Fig. 2.5A) or free T_4 fraction (Fig. 2.5B). Moreover, hepatic uptake and free T_4 fraction with OA was significantly reduced compared to that of both PFOA and PFOS.

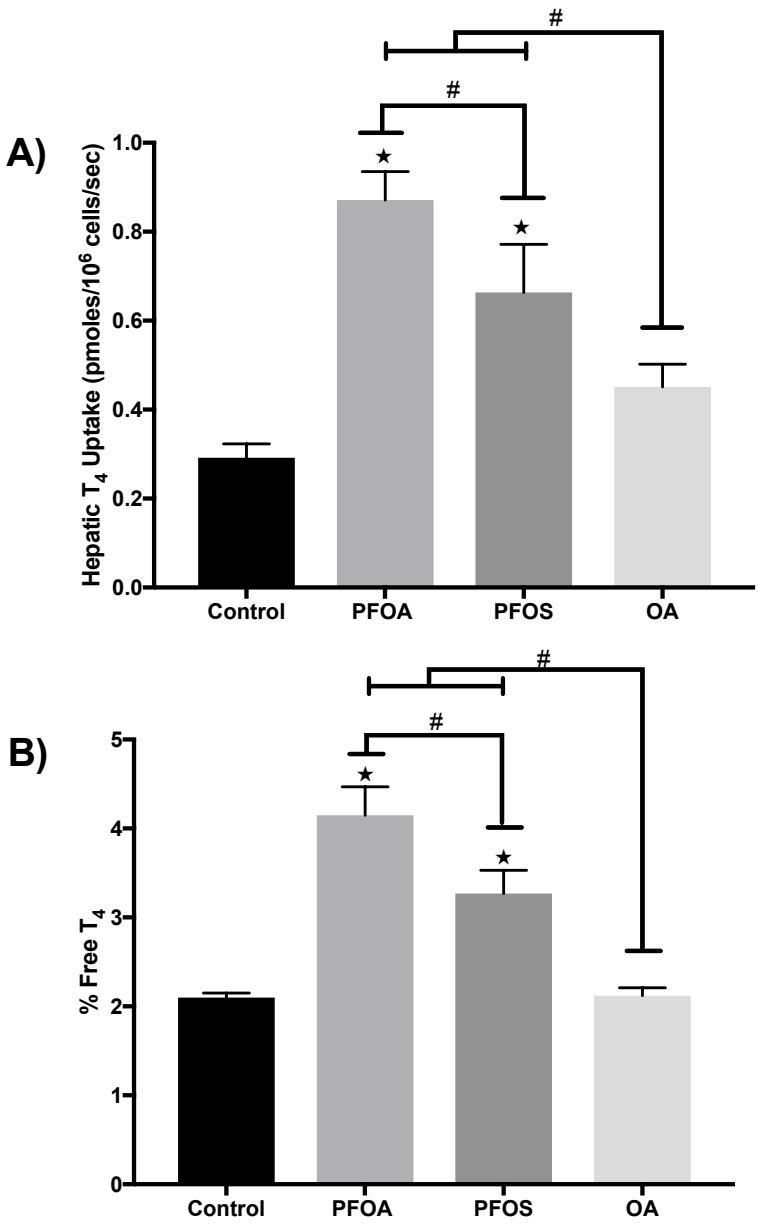

Figure 2.5: PFAS Impact on T_4 Uptake. (A) Hepatic T_4 (1 μM) uptake was evaluated with PFOS (10 μM) and OA (10 μM) in comparison to PFOA (10 μM) with TTR (62.5 μg/mL) present. Data represents uptake rate determined from the slope of linear regression (pmoles/10^6 cells/sec) ± SD from n = 3 independent experiments. (B) The free T_4 fraction (%) was evaluated with PFOS (10 μM) and OA (10 μM) in comparison to PFOA (10 μM) with TTR (62.5 μg/mL) present. Data represents mean ±SD from n=3 independent experiments. (★) represents a statistically significant difference ($p<0.05$) compared to 37°C control, (★★) represents a statistically significant difference ($p<0.05$) compared to 4°C control, (★★★) represents a statistically significant difference ($p<0.05$) between 37°C and 4°C, (#) represents a statistically significant difference ($p<0.05$) between samples within a temperature range. Data was analyzed with a one-way ANOVA with a Tukey's post-hoc test.

2.4 Discussion:

T_4 carrier-mediated transport was successfully characterized by analyzing uptake kinetics and inhibiting T_4 transport with CsA. Significant differences in T_4 uptake between 37°C and 4°C controls also suggests the presence of a temperature-dependent uptake process. This finding is in contrast to some previous reports suggesting passive diffusion to be the primary mode of T_4 uptake (Rao & Rao 1983, Nicoloff *et al.* 1981). This discrepancy may be related to differences in procedural methods. In a report investigating the ability of T_4 uptake to regulate T_3 production, a concentration of over 30 μM T_4 was used to ensure sufficient substrate availability (Nicoloff et al. 1981). In relation to kinetic findings herein, this concentration is well above the estimated K_m of hepatic T_4 transport. Therefore, saturation of T_4 transport would suggest passive diffusion to be predominant. An additional report characterizing T_4 uptake in liver cells as passive diffusion uses a method similar to our own. However, the use of radiolabeled T_4 may overestimate the amount of T_4 uptake due to inability to delineate between parent and metabolite compounds (Rao & Rao 1983). In contrast, the LC-MS/MS method used herein may more accurately detect transport of the parent T_4 species leading to differing results.

We acknowledge that CsA can impact the activity of efflux transporters that may be present in cryopreserved hepatocytes (Wang *et al.* 2008). It is possible that CsA demonstrates some impact on substrate efflux of T_4 in our uptake model that would result in an increase in intracellular T_4 concentrations. However, due to the decrease in T_4 accumulation with increasing CsA concentrations in our data, we suggest that the impact of CsA is principally to inhibit T_4 uptake into our uptake model.

PFOA carrier-mediated uptake has previously been characterized in isolated rat hepatocytes with an estimated K_m of 88 μM (Han et al. 2008). While measuring the uptake rate of PFOA was not in the scope of this project, the potential inhibition of T_4 uptake by PFOA was of interest. Herein we demonstrate that PFOA does not inhibit T_4 uptake in isolated rat hepatocytes within the concentration levels tested. It is possible that T_4 may have a higher affinity for hepatic transport demonstrated by comparing the K_m of transport for T_4 defined herein (5.05 μM) to that of PFOA (88 μM) (Han et al. 2008). It is also possible that PFOA may be transported into hepatocytes via different transporters than T_4 or that different binding sites for PFOA and T_4 may be present on the same transporters.

Changes in the free T_4 fraction caused by varying the concentration of TTR demonstrated significant alterations in the rate of hepatic T_4 uptake. This finding suggests that the rate of T_4 uptake is dependent on the free T_4 fraction. Similar findings have been demonstrated previously, suggesting that the uptake of highly protein bound substrates are dependent upon the substrate's free fraction (Amaraneni et al. 2017, Amaraneni et al. 2016). This finding also acts as a basis for our hypothesis that PFAS compounds will be able to impact T_4 uptake by altering the free T_4 fraction.

The effect of PFOA and PFOS on the free T_4 fraction is well documented, however the impact of this alteration on TH uptake had not previously been demonstrated. Herein, PFOA and PFOS cause an increase in T_4 uptake as a consequence of displacement from TTR. Furthermore, due to the prevalence of carrier-mediated T_4 transport, we suggest that this increase in uptake is primarily driven by carrier-mediated uptake processes. This is supported by a correlation between increasing free T_4 fraction and hepatic T_4 uptake at

37°C and the absence of correlation at 4°C. Significant differences were also identified in the uptake of T_4 between 37°C and 4°C at PFOA concentrations of 5, 8, and 10 μM. This adds further evidence that a temperature specific response to PFOA is largely a result of carrier-mediated T_4 transport.

Increased biliary excretion of THs has been identified as a pathway by which PFASs cause a decrease in systemic TH concentrations (Chang et al. 2008). The role of hepatic transporters in xenobiotic and endobiotic disposition and excretion has also been identified at length (Kovacsics *et al.* 2017, Zaher *et al.* 2008). Findings that PFOA and PFOS result in increased carrier-mediated T_4 transport could provide an additional mechanistic step involved in increasing TH excretion and lowering systemic TH concentrations *in vivo.* This is further supported by reports of TTR null mice being subject to a significant decrease in systemic total T_4 when compared to their wild type counterparts (Palha et al. 1994, Episkopou *et al.* 1993). This increase in T_4 excretion is consistent with that observed PFAS treated rats adding further evidence that a change in free T_4 fraction leads to subsequent excretion.

In conclusion, PFOA and PFOS are demonstrated to cause a significant increase in hepatic T_4 uptake. This impact is demonstrated to be a result of PFAS alteration in free T_4 fraction and the presence of carrier-mediated T_4 transport. Future research will be necessary to evaluate contributions of hepatic transport proteins for T_4 uptake to evaluate specific impacts of PFAS compounds.

CHAPTER 3

Species Comparison of Hepatic OATP T_4 Transport

3.1 Introduction:

Hepatocytes co-express a wide variety of transmembrane transport proteins responsible for the movement of endogenous and exogenous compounds into the liver for metabolism (Burt et al. 2016, Hallifax & Houston 2006). The transporters expressed in the liver often have overlapping substrates, making it difficult to determine substrate specificity for specific transporters. THs are no exception, as they are characterized substrates for members of the OATP, NTCP, OAT, and MCT transporter families localized in hepatocytes (Visser et al. 2011).

The most abundantly expressed family of transporters in the liver are OATPs. In humans OATP1B1, 1B3, and 2B1 make up nearly 70% of hepatic transporter expression (Burt et al. 2016). However, OATP1B1 in particular is demonstrated to be the predominant protein expressed in the liver for substrate uptake (Kimoto *et al.* 2012, Burt et al. 2016). The rodent orthologue, Oatp1b2, is also found to be the most abundantly expressed transporter in rodent liver (Sidler Pfandler *et al.* 2004, Cattori *et al.* 2000). There is evidence that THs act as substrates for both OATP1B1 and Oatp1b2, however the contributions of these proteins to total T_4 uptake is unknown (Visser et al. 2011, Cattori *et al.* 2001, Hagenbuch & Meier 2004).

In Chapter 2 the impact of PFAS compounds on TH uptake was established. PFOA and PFOS demonstrate the ability to increase hepatic T_4 uptake in the presence of

serum binding proteins, while not demonstrating an inhibitory impact at the site of transport. Furthermore, we infer that this impact is caused primarily by carrier-mediated transport. The limitations of hepatocytes render evaluation of PFAS impacts on specific transporters challenging. In order to further evaluate hepatic T_4 transport, it is necessary to evaluate uptake in the predominantly expressed liver specific transporters.

The transporter protein MCT8 described in Chapter 1 is particularly important transport protein due to its predominant expression in neurons and high specificity for T_4 transport (Visser et al. 2011). Interestingly, MCT8 has also been identified as a T_4 transport protein in the liver (Friesema et al. 2003). There is currently no direct quantitative comparison in expression between MCT8 and OATPs. However, the lack of quantitative comparison could suggest MCT8 makes up a much smaller fraction of transporter expression than other hepatic transporters (Burt et al. 2016). While it would be beneficial to identify the impacts of PFASs on T_4 uptake mediated by MCT8 transport, identification of the impacts of PFAS on T_4 transporters known to have quantitatively predominant expression in hepatocytes such as OATP1B1 and Oatp1b2 would allow for the assessment of potential implications on T_4 excretion.

The present study evaluated OATP1B1 mediated T_4 transport with the use of OATP1B1 overexpressed HEK293 cells. T_4 uptake via rodent Oatp1b2 was also evaluated to provide a species comparison of hepatic T_4 transport. This evaluation provides evidence of transporter specific OATP1B1 and Oatp1b2 uptake that could be a basis for future analysis regarding how xenobiotics could impact specific transporter kinetics. Our hypothesis for this work is that uptake of T_4 is mediated by both OATP1B1 and Oatp1b2 transporters. The first aim of this research will be to demonstrate T_4 uptake

in OATP1B1 transfected HEK293 cells. The second will be to develop a species comparison by evaluating T_4 uptake in rodent Oatp1b2 transfected HEK293 cells. The findings from this study will provide the groundwork for future analysis of PFAS mediated effects on specific hepatic T_4 transporter kinetics.

3.2 Methods:

3.2.1 Chemicals and reagents

L-Thyroxine (T_4; (2S)-2-amino-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5diiodo-phenyo] propanoic acid), (T_3; (2S)-2-amino-3-[4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl] propanoic acid), cyclosporine A (CsA), Estradiol-17b-glucuronide, and aldostrone-D_4-glucuronide were purchased from Sigma (St. Louis, MO). $^{13}C_6$-labeled T_4 ($^{13}C_6$-T_4) was purchased as an internal standard from Cambridge Isotope Laboratories (Tewksbury, MA). DMEM cell culture media, Hank's Balance Buffer Solution (HBSS), MEM nonessential amino acids, penicillin-streptomycin, 0.25% trypsin, OATP1b2 transfected, OATP1B1*1a transfected, and WT control Transportocells were purchased from Corning (Corning, NY). Fetal bovine serum albumin was purchased from VWR (Radnor, PA). Sodium butyrate was purchased from Alfa Aesar (Wardhill, MA). Ecolite(+) scintillation cocktail was purchased from MP Biomedicals (Santa Ana, CA). [^{3}H]-E3S (50 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO) All solvents and acids used in extraction and elution were of Optima-Grade performance and purchased from Thermo Fisher (Pittsburgh, PA).

3.2.2 Uptake assay

Cell culture

Cryopreserved HEK293 cells transfected with the *SLCO1B1*, *Slco1b2*, or empty vector pcDNA gene were plated in T-175 flask at a seeding density of 250,000 cell/cm^2 using DMEM culture media supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% MEM nonessential amino acids. Cells were incubated overnight in a cell culture incubator at 37°C and 5% CO_2. 24 hours after initial plating, media was replaced and supplemented with 2 mM sodium butyrate and incubated for an additional 24 hours before use in uptake studies.

Uptake assay

Cultured HEK293 cells were brought to a concentration of $1.0x10^6$ cells/mL in HBSS buffer at 37°C by cell counting with trypan blue exclusion. Aliquots of $0.5x10^6$ cells were divided into individual groups. Uptake was evaluated by incubating 5.0×10^5 cells for 5 minutes with T_4 (0.1 µM), [^{3}H]-estrone-3-suflate ([^{3}H]-E3S; 20 pM), or estradiol-17b-glucuronide (E17bG; 2 µM), with or without transport inhibitor cyclosporine A (CsA; 10 µM). Uptake was terminated with the addition of an equal volume of ice-cold HBSS buffer and centrifugation of cells at 2352xg (Hettich MIKRO220) for 30 sec. The resulting supernatant was removed, and cells were re-suspended in ice-cold HBSS buffer before re-centrifugation at 2352 x g for 30 sec. After supernatant was removed, 80% ACN with or without internal Standard ($^{13}C_6$-labeled T_4 or A-D_4-G) was added to lyse cells and precipitate protein before centrifugation at 18440xg for 10 min. Resulting supernatant was collected for LC-MS/MS or scintillation analysis. The remaining pellet was re-solubilized

with 100 mM sodium hydroxide for protein quantification. All uptake data was normalized to protein using BCA protein quantification.

3.2.3 Analysis

E3S Analysis:

Aliquots of supernatant collected from uptake assays conducted with E3S was added to 2 mL of Ecolite(+) scintillation cocktail(PerkinElmer Life and Analytical Sciences), and the total radioactivity was measured with a Beckman Coulter (Fullerton, Ca) LS6500 Scintillation counter. LSC was set to read ^{3}H for 2 mins. Data was recorded in DPM and converted to pmoles before normalization to protein.

T_4 Analysis:

T_4 and T_3 were analyzed by LC-MS/MS following procedures described in Chapter 2.

E17bG Analysis:

Samples were analyzed for E17bG using an Agilent 1200 Ultra-performance liquid chromatograph (UPLC) coupled to a 6420 triple quad mass spectrometer (Agilent, Santa Clara, CA). Injections (5 uL) at a 0.3 ml/min flow rate were made onto an Agilent Zorbax XDB-$_{C18}$ column (4.6 mm x 50 mm, 1.8 um particle diameter; Santa Clara, CA) maintained at 40˚C. Gradient elution with methanol (solvent A) and water (solvent B) containing 0.2% FA was applied under the following conditions: 70% A for 0 min, followed by a linear gradient to 70% A at 0.5 min, increasing to 80% A at 5.0 min, and held for a 20 min stop

time. The column was then allowed to re-equilibrate under the original conditions for a 3 min post-time. MS/MS detection was conducted using ESI- in multiple reaction mode under the following conditions: E17bG quantifying transition ion *m/z* 447.3-271.4 (collision energy 25V), with qualifying ion transitions *m/z* 447-85.1 (collision energy 30V), with the fragmenting voltage set to 135 V and the cell accelerator at 7 V. The internal standard, androsterone-D_4-glucuronide (A-D_4-G) was quantified based on the transition ion *m/z* 469.3-113.1 with the fragmenting voltage, collision energy, and cell accelerator set to 135 V, 30 V, and 7 V, respectively (Wang and Stapleton, 2010). ESI source parameters were applied according to the following: source gas temperature 350˚C, gas flow 11 L/min, nebulizer 55 psi, capillary 4000 V. E17bG standard curves (0.1–125 ng/mL) were prepared in (80:20 ACN:H2O) using A-D_4-G as an internal standard and verified during analysis with a check standard every 12 samples, followed by a blank sample for carryover assessment. Data processing was performed using Agilent MassHunter software (version B.04.01) and sample concentrations were determined using an internal-standard response factor.

3.2.4 Statistical analysis

Experiments were performed using 3 separate groups of aliquots assayed individually from a single vial of cryopreserved transfected cells purchased from Corning (Corning, NY). Assay results are presented as mean ± S.D. Statistical significance was evaluated between groups using two-way ANOVA analysis with Tukey's post hoc test or student's t-test, using Graphpad Prism 7 software.

3.3 Results:

3.3.1 Characterization of Uptake Model

Functional characterization of HEK293 transport models transfected with *SLCO1B1*1a* and *Slco1b2* gene sequences were conducted with well characterized substrates for 1B1 and 1b2 uptake. A significant increase in uptake of E3S was demonstrated in OATP1B1*1a transfected HEK293 cells compared to the vector control (Fig. 3.1A). Similarly, E17bG uptake was significantly increased in HEK293 cells transfected with Oatp1b2 gene sequence compared to the vector control (Fig. 3.1B). To further demonstrate functionality of transport, the well characterized transport inhibitor CsA was added. A significant decrease in both transfected HEK293 cells was demonstrated with the addition of CsA in comparison to their vector controls (Fig. 3.1A and B). Furthermore, the addition of CsA decreased the uptake of both compounds to that of the vector control, as no significance between vector control and overexpression was detected (Fig. 3.1A and B).

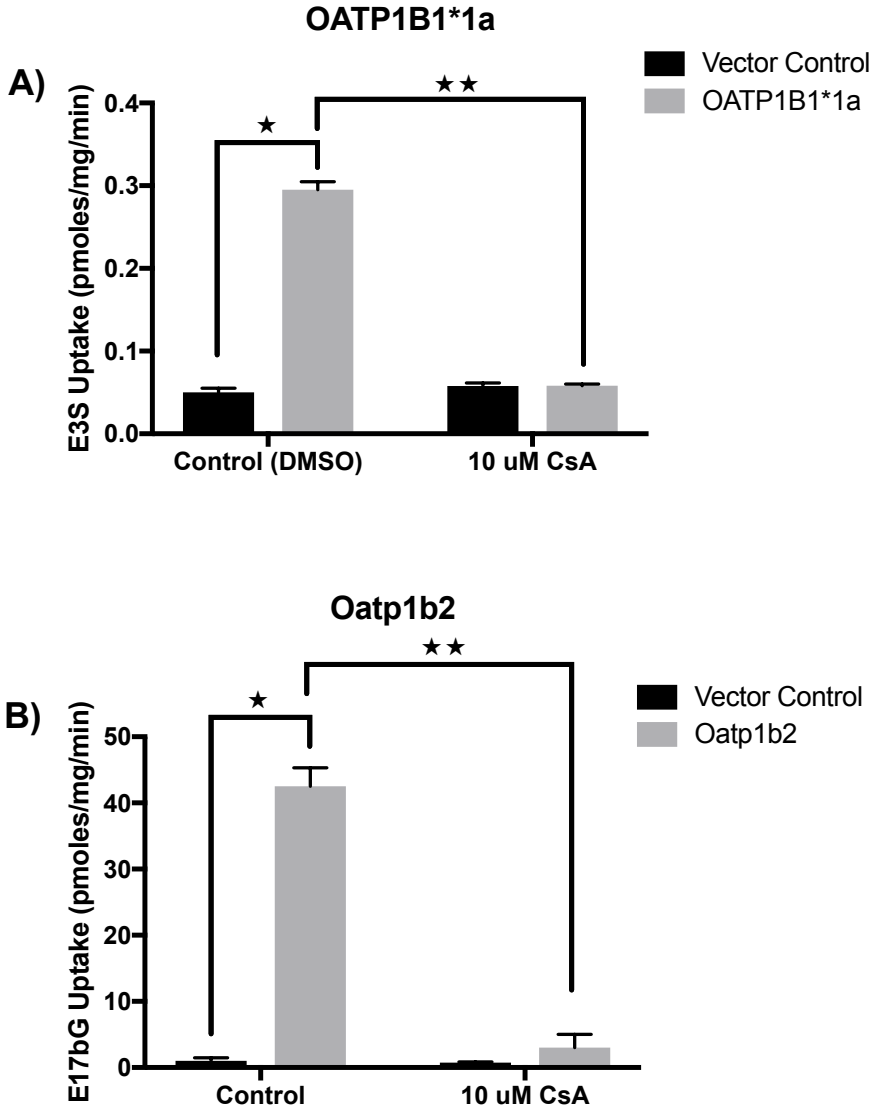

Figure 3.1. Functional Transport Characterization. (A) E3S (20 pM) uptake rate in OATP1B1*1a transfected HEK293 cells and vector control pcDNA HEK293 cells after incubation for 5 minutes with vehicle control (DMSO) or 10 uM cyclosporine A (CsA). Data represents mean ± SD (n=3). (B) E17bG (2 μM) uptake rate in OATP1B1*1a transfected HEK293 cells and vector control pcDNA HEK293 cells after incubation for 5 minutes with vehicle control (DMSO) or cyclosporine A (10 μM). Data represents mean ± SD (n = 3). (★) represents statistically significant difference ($p < 0.05$) compared to vector control. (★★) represents statistically significant difference ($p<0.05$) compared to SLCO transfected cell line. Statistical analysis was preformed using two-way ANOVA with a Tukey's post-hoc test.

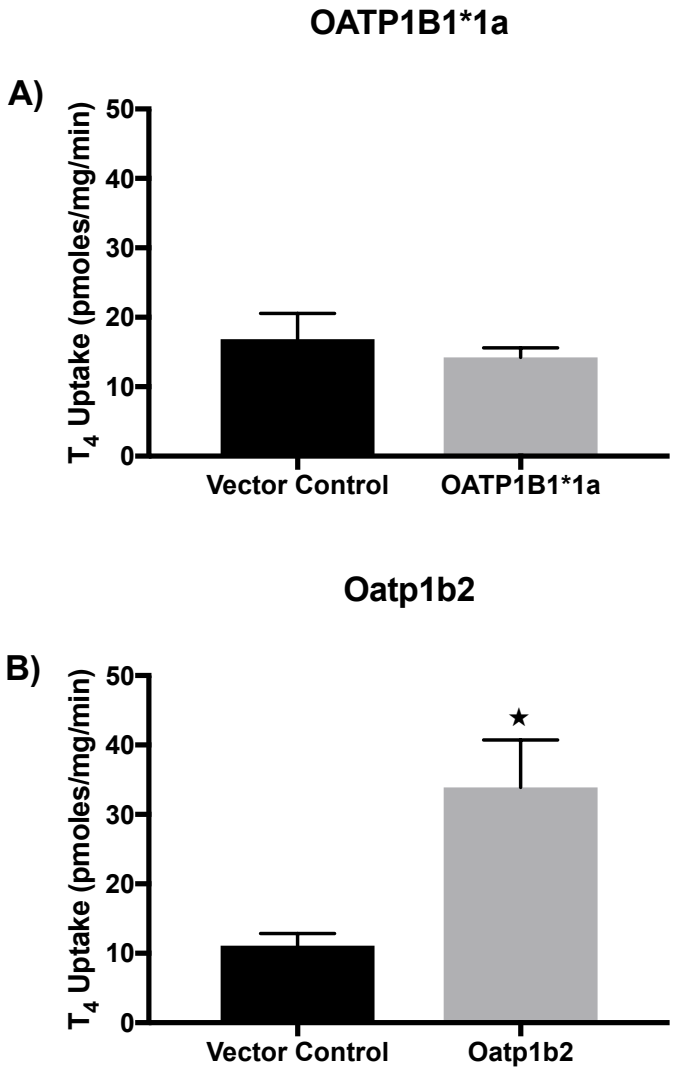

Figure 3.2. OATP T_4 Transport. (A) T_4 (0.1 μM) uptake rate in OATP1B1*1a transfected HEK 293 cells and vector control pcDNA HEK293 after incubation for 5 minutes with vehicle control (DMSO). Data represents mean with error bars denoting ± SD (n = 3). (B) Thyroxine uptake (0.1 μM) in Oatp1b2 transfected HEK293 cells and vector control pcDNA HEK293 cells after incubation for 5 minutes with vehicle control (DMSO). Data represents mean with error bars denoting ± SD (n = 3). (★) Statistically significant difference in T_4 uptake compared to vehicle control ($p < 0.05$). Statistical analysis was conducted using student's t-test.

3.3.2 T_4 Transport in Overexpression Models

Uptake of T_4 was conducted at physiological serum levels of 0.1 μM. A significant increase in T_4 transport was not detected in OATP1B1*1a overexpressed HEK293 cells (Fig. 3.2A). However, a significant increase in T_4 uptake rate was demonstrated in Oatp1b2 overexpressed HEK293 cells when compared to vector control (Fig. 3.2B).

3.4 Discussion:

The evaluation of T_4 transport in an OATP1B1 overexpressed cell line was chosen due to the predominant hepatic expression of OATP1B1 (Burt et al. 2016, Tamraz *et al.* 2013, Visser et al. 2011, Abe *et al.* 1999). However, our study did not observe T_4 to be a substrate for this transporter. This finding is in contrast to previous reports suggesting T_4 may be a substrate for OATP1B1. In its initial characterization, Abe *et al.* demonstrated saturation of T_4 uptake in OATP1B1 overexpressed oocytes. Kinetics for T_4 transport in this report estimated a K_m of 3.0 μM, however comparison to a passive diffusion component was not evaluated (Abe et al. 1999). A potential source for discrepancy in these results may be due to differences in the experimental models. Oocytes provide a transfection model more ideal for evaluating substrate transport than HEK293 cells. This is due to differences in endogenous expression of transporters. HEK293 cells express more endogenous transporters than oocytes, leading to possible non-specific transport of substrate (Ahlin *et al.* 2009). Differences in substrate transport between the two models have been noted previously. A study comparing uptake in both models noted a difference in the presence of saturable transport of pravastatin between HEK293 and oocytes transfected with the *SLCO1B1* gene (Ulvestad 2007).

An additional reason for these conflicting reports may be the amount of genetic variation of the OATP1B1 protein. To date, 54 different polymorphic genetic variants have been identified as a result of genetic shifts in different locations along the *SLCO1B1* gene sequence (Tamraz et al. 2013). Of those currently identified, a collection of specific haplotypes have been identified to present clinical relevance (Maeda *et al.* 2006, Gong & Kim 2013). This is due to a significant impact on transport activity and high allelic frequency in certain populations (Pasanen *et al.* 2008, Tamraz et al. 2013). One example is the haplotype OATP1B1*1b, which is found in 39% of the American population (Pasanen et al. 2008). This haplotype demonstrates a significant change in the transport affinity for pravastatin leading to a decrease in AUC compared to the *1a haplotype (Maeda et al. 2006). Other clinically significant haplotypes include *5, *14, *15, *17, all of which demonstrate differing affinity for transport substrates (Tamraz et al. 2013, Kim *et al.* 2007, Pasanen et al. 2008). In the current study the reference haplotype (OATP1B1*1a) was used, however previous reporting does not state which haplotype was used (Abe et al. 1999). it is possible that other genetic variants may have been used that impacted functional activity of T_4 transport.

In contrast to our findings in OATP1B1, we demonstrate that T_4 is a substrate for the rodent orthologue Oatp1b2. This finding agrees with previous reports characterizing Oatp1b2 as a transporter for T_4. This species related difference in substrate specificity is not unprecedented. While OATP1B1 and Oatp1b2 share 64% sequence homology and many overlapping substrates, other examples of interspecies differences in these transporters have occurred. Specifically, a difference in transport affinity for sorafenib

noted *in vitro* demonstrates sorafenib transport by OATP1B1 and a lack of transport by Oatp1b2 (Zimmerman *et al.* 2013).

While we were not able to demonstrate T_4 substrate specificity for OATP1B1*1a, it is possible that T_4 transport could occur in a different OATP1B1 haplotype. If this is indeed the case, it is possible that a subset of the population could be more sensitive to PFAS impacts characterized in Chapter 2. More specific research of T_4 uptake in OATP1B1 haplotypes will be necessary to identify such events. OATPs represent a contribution to overall hepatic T_4 uptake. It is possible that other transporters localized in hepatocytes may contribute to T_4 uptake as well. Therefore, further analysis of additional hepatic transporters should be considered to gain a better understanding of T_4 hepatic uptake. The findings of this study serve as the initial groundwork for evaluating how PFAS compounds are able to impact specific T_4 transporter kinetics.

CHAPTER 4

Summary and Future Directions

4.1 Summary:

The impact of PFAS compounds on systemic TH concentrations has been well documented in both animal models and human epidemiological studies (Martin et al. 2007, Chang et al. 2008, Coperchini et al. 2017, Yu et al. 2009, Weiss et al. 2009). The purposed underlying mechanism of PFAS action on the HPT axis consists of a change in TH excretion by altering the bound fraction of TH in serum, and up-regulating metabolic deactivation of TH leading to excretion (Chang et al. 2008, Yu et al. 2009, Weiss et al. 2009). However, it was not previously understood how PFAS compounds might impact TH uptake into the cell, particularly through carrier-mediated transport mechanisms. This is of importance as TH uptake can act as a rate limiting step of TH metabolism by influencing the intracellular concentration of THs (Hennemann et al. 2001). There is evidence to suggest PFAS compounds could inhibit TH uptake due to the role of transporters in the uptake and distribution of various PFAS compounds (Han et al. 2008, Zhao et al. 2017). There is also reason to expect PFASs might increase TH uptake due to previous work demonstrating how protein binding impacts carrier-mediated transport (Amaraneni et al. 2017, Amaraneni et al. 2016, Mendel 1989). Given the role of TH uptake as a rate-limiting step of TH excretion, it was imperative to evaluate how PFASs could impact TH uptake mechanisms (Hennemann et al. 2001).

Our hypothesis for this work stated that PFAS compounds would be able to alter TH uptake. To determine the impacts of PFASs on TH uptake we evaluated the impact of two widely distributed PFAS compounds, PFOA and PFOS, on TH uptake in cryopreserved rat hepatocytes in the presence and absence of the serum protein TTR. Our findings suggested that PFOA does not inhibit T_4 uptake. However, PFOA and PFOS are able to increase the rate of TH uptake by displacing T_4 from the serum binding protein TTR. We also speculate that this impact on hepatic T_4 uptake is ultimately a result of an increase in carrier-mediated T_4 transport. This finding agrees with previous reports demonstrating that altering the free and bound fraction of substrate can impact the rate of substrate uptake (Amaraneni et al. 2017, Amaraneni et al. 2016, Mendel 1989).

To develop a better understanding of hepatic T_4 transport, we evaluated T_4 uptake by predominant liver specific transporter OATP1B1*1a (Hagenbuch & Meier 2004). However, in our analysis we found that T_4 was not a substrate for this transporter. This finding is in contrast to previous reports characterizing T_4 as an OATP1B1 substrate (Abe et al. 1999, Tamraz et al. 2013, Hsiang *et al.* 1999). We can confirm from our study that the OATP1B1*1a transporter was functional, as the probe substrate, E3S, demonstrated sufficient OATP1B1 activity. To relate this finding to our previous work conducted in rat hepatocytes, we also evaluated T_4 uptake in the rodent OATP1B1 orthologue, Oatp1b2 (Cattori et al. 2000, Cattori et al. 2001). We found T_4 to be a substrate of Oatp1b2, further confirmed by uptake of the probe substrate E17bG. This finding is concurrent with reports demonstrating T_4 to be a substrate of Oatp1b2. These findings also suggest a species difference in substrate specificity between the liver specific human OATP1B1 and its

rodent orthologue Oatp1b2 that shares 64% sequence homology (Hagenbuch & Meier 2004).

The significance of our finding that PFOA and PFOS are able to increase the hepatic uptake rate of T_4 lies in the mechanistic understanding of how PFAS alters TH pharmacokinetics. Our findings provide further evidence that PFOA and PFOS displace T_4 from TTR. We expand upon this by providing further mechanistic insight that suggests a subsequent increase in hepatic uptake of T_4 occurs following displacement. This increase in uptake could result in more TH available for metabolic deactivation and excretion, thereby decreasing systemic TH concentrations. This effect could also work in concert with the reported PFAS induction in TH metabolic enzymes, further exacerbating their impact on TH serum concentrations. We suggest that the change in serum concentrations of THs caused by increased TH metabolism and excretion would result in less TH available to other tissues in the body that need THs to function properly. The physiological impact of this phenomenon may result in a hypothyroid state at the tissue level leading to symptoms similar to that of hypothyroidism.

Given the differing classifications of hypothyroidism, it is difficult to suggest how the impacts of PFAS compounds on serum concentrations may be clinically classified. Interestingly, in animal models the majority of cases where PFAS lowers THs, it does not result in a concomitant rise in TSH (Chang et al. 2008, Martin et al. 2007). In these cases, the more precise diagnosis for this impact would be ‘hypothyroxinemia’, which is marked by a decrease in systemic free T_4 without a rise in TSH (Dosiou & Medici 2017, Chang et al. 2008). While hypothyroxinemia is able to cause symptoms similar to hypothyroidism, the majority of its prevalence and adverse health impacts are noted during pregnancy

with little reported on effects in the general population (Dosiou & Medici 2017, Haddad 2008). The impact of hypothyroxinemia during pregnancy usually results in irreversible damage to fetal neurons, as the fetus is dependent upon the mother's thyroid hormones throughout the pregnancy term. In particular, reports have found that maternal hypothyroxinemia is associated with a higher risk of developing ADHD, schizophrenia, low I.Q., decreased motor function, and increased occurrence of autism (Roman et al. 2013, Henrichs et al. 2013, Li et al. 2010, Pop et al. 2003, Gyllenberg et al. 2016). However, Low systemic T_4 levels without a change in TSH can still be classified as a form of subclinical or overt hypothyroidism if a patient is presenting signs concurrent with hypothyroidism (Haddad 2008). Therefore, PFASs may contribute to cases of thyroid diseases classified as either hypothyroxinemia or hypothyroidism.

The level of PFOA an PFOS deemed to have an impact on T_4 uptake in our analysis are at levels higher than those typically identified in the general population (Winquist & Steenland 2014, Knox *et al.* 2011). Doses of both PFOA and PFOS did not demonstrate a significant effect until reaching concentrations above 5 μM. These levels of PFOA and PFOS have been detected in individuals occupationally exposed to PFOA and PFOS (Olsen & Zobel 2007, Olsen et al. 2007). Therefore, those occupationally exposed may be at a higher risk for developing a PFAS mediated TH effects. Given the higher rate of thyroid toxicosis in women, it is also possible that women who work with PFAS compounds may be the most at-risk population for developing a PFAS related toxic event (Haddad 2008).

The findings of our second study suggest a species related difference causes a change in carrier-mediated TH uptake. Since our hypothesis is centered around the

impact of PFASs on carrier-mediated TH uptake, the effects seen in rodent hepatocytes may not be as demonstrable in humans. Further studies conducted with human hepatocytes or with other liver specific transporters will be necessary to evaluate what impact PFAS compounds have on human TH uptake.

In conclusion, we have demonstrated the ability of two PFAS compounds, PFOA and PFOS, to increase hepatic T_4 uptake. This finding is novel and adds additional mechanistic insight to support reports of PFAS lowering systemic TH levels *in vivo*. We suggest that this impact is primarily due to an increase in carrier-mediated T_4 uptake following displacement from serum proteins. We also observed a species difference in T_4 transport specificity between human OATP1B1*1a and rodent Oatp1b2. This finding suggests more work is necessary to understand how PFAS compounds may impact human T_4 transport.

4.2 Experimental Limitations:

In vitro uptake models

In vitro models are commonly used to identify specific mechanistic interactions that may lead to physiological impacts. In the context of the present study, we were interested in evaluating the impact of PFAS compounds on the uptake of T_4 as a potential mechanism of PFAS mediated reductions in serum T_4 identified *in vivo*. To investigate this, we utilized cryopreserved rodent hepatocytes to evaluate the uptake of T_4 in the presence of PFAS compounds. While we were able to demonstrate an increase in T_4 uptake occurs following PFAS displacement of T_4 from serum proteins, the *in vitro* techniques used herein do not allow for concrete extrapolation to an *in vivo* system.

Therefore, we draw assumptions that the increase in uptake could be responsible for the decrease in systemic THs previously identified *in vivo* (Chang et al. 2008, Martin et al. 2007). Further, we are not able to evaluate how a potential decrease in systemic THs could lead to a hypothyroid state in various tissues in the body or how the HPT-axis may correct for losses in THs over time. To overcome these limitations, future work will aim to evaluate the *in vivo* implications of TH loss in serum due to an increase in hepatic T_4 uptake.

Hepatocytes suspension uptake model

In this study cryopreserved primary rat hepatocytes were used in a suspension assay to evaluate T_4 uptake. The benefit of this model is the functional expression of many transporters to mimic the expression of hepatocytes *in vivo.* Uptake studies were conducted in suspension to eliminate loss in transporter expression caused during plating of hepatocytes (Richert *et al.* 2006). However, some limitations are associated with the use of this technique. As previously noted, it is not possible to delineate the contributions of specific transporters to substrate uptake, therefore the impact seen in this study in the presence of PFAS compounds must be viewed as a cumulative effect instead of addressing which transporter(s) may be responsible for the impact identified. Additionally, evaluating substrate uptake in suspension makes it difficult to evaluate the efflux of compounds that may also be in occurrence. We were not able to identify what impacts PFAS compounds may have on the efflux of T_4 from the hepatocytes. To overcome these limitations, we will aim to evaluate the impacts on specific transport proteins and in models more conducive to evaluating the impact of PFAS on T_4 efflux.

LC-MS/MS analysis

The use of a non-radiolabeled analytical methods provides benefits of reducing hazards associated with radioligand procurement and use. LC-MS/MS analysis specifically allows for the detection and quantification of multiple analytes or metabolic products. This was particularly advantageous to this project given the propensity for T_4 metabolic conversion. However, the use of LC-MS/MS analysis does have quantification limitations in comparison to detection of isotopically labeled compounds (Jayarama-Naidu et al. 2015). This limitation did present difficulties in evaluating T_4 uptake in our analysis. Notably, the concentrations of T_4 (1.0 μM) and TTR (62.5 μg/mL) used herein were optimized to allow for accurate detection and are not consistent with human physiological levels (0.1 μM; 250 μg/mL). This could present difficulty in extrapolating the work to more human relevant physiological conditions. To overcome this limitation in future experiments, we will aim to evaluate this interaction with more sensitive detection methods to more accurately evaluate the impacts of PFAS compounds on displacement and uptake of T_4.

4.3 Future Directions:

Impact of PFAS on specific transporters

The exact impact PFAS compounds have on transporter kinetics is still unknown. In Chapter 3 of this work, an attempt was made to investigate the impacts of PFAS compounds on the liver specific transporter, OATP1B1*1a, due to its prevalent expression in human hepatocytes and reported selectivity for T_4 (Burt et al. 2016, Abe et al. 1999). However, this work revealed that T_4 is not a substrate of OATP1B1*1a. While we were

not able to demonstrate transporter specific kinetic changes with OATP1B1*1a, it is likely that one or more additional transporters also available in the liver is responsible for uptake of T_4 into hepatocytes. It would behoove future research to investigate the impact of PFASs on other transporters to gain a better understanding of how the kinetics of T_4 transport may be altered by displacement of T_4 from serum proteins.

Furthermore, the impact of PFASs on TH transporter uptake in other tissues would be of considerable interest. Two key TH transporters localized in the brain are MCT8 and OATP1C1 (Visser et al. 2011). Genetic defects of both MCT8 and OATP1C1 are demonstrated to have a substantial impact on thyroid status as well as organ functionality, highlighting the importance of both transporters (Visser et al. 2011, Brozaitiene *et al.* 2018, Fuchs *et al.* 2009). The scope of the current study was focused on the impacts of PFASs on hepatic T_4 uptake, to which MCT8 and OATP1C1 are not known to play a large role. However, the potential impact of an increase in T_4 uptake via MCT8 and OATP1C1 in the brain presents the potential for a hyperthyroid tissue status that is worth consideration. Future research could benefit from evaluating the impacts of PFASs on T_4 uptake in MCT8 and OATP1C1.

Use of Human Hepatocytes

Cryopreserved rat hepatocytes were chosen as uptake models due to both cost and limit of inter-donor variability between samples when compared to human hepatocytes (Hallifax & Houston 2006). As we demonstrated in the third chapter of this project, there seems to be a considerable species difference in the uptake of T_4 between rodent and human OATP1B1 and Oatp1b2 orthologues. As we pose in our first aim, the impact of PFAS on T_4 uptake is primarily driven by carrier-mediated process. However, If

human hepatic T_4 uptake is less dependent on carrier-mediated transport, the impact of PFASs may not be as prominent in humans. The easiest way to determine if PFASs do, in fact, have an impact on human hepatic T_4 uptake would be to use the current uptake methodology suggested in Chapter 2 with cryopreserved human hepatocytes. This adaptation in assay system should be a simple change allowing for a direct comparison of rodent and human hepatocytes, as well as providing more conclusive evidence to the impacts of PFAS compounds on human hepatic T_4 uptake.

Impact of other PFAS compounds

While PFOA and PFOS are the most widely distributed and studied PFAS compounds, regulations and safety concerns have led to a decline in PFOA and PFOS manufacturing (Prevedouros et al. 2006, Ahrens & Bundschuh 2014). Following this decline, a subsequent decrease in human exposure has occurred in the U.S. However, due to the commercial demand for PFAS products, manufacturers have shifted their focus to the use of shorter PFAS carbon chains such as PFHxS and PFBS (Krafft & Riess 2015, Ahrens & Bundschuh 2014). Certain manufacturers have also developed new alternative compounds to replace the effective PFOA and PFOS. The new 'GenX' process includes numerous perfluorinated compounds with many physicochemical similarities to the aforementioned eight-carbon PFAS compounds. The impacts of the new GenX compounds is still relatively unreported due to their relative infancy compared to their predecessors (Heydebreck *et al.* 2015). However, the shorter chain PFHxS and PFBS have shown some toxic impacts in both animals and humans (Sundstrom *et al.* 2012, Environment 2015). PFHxS in particular has demonstrated a similar affinity for blood serum and ability to evade biochemical degradation while also demonstrating an ability

to displace T_4 from its serum binding proteins (Weiss et al. 2009, Ren et al. 2016, Olsen et al. 2007).

While understanding the role of how PFOA and PFOS lower systemic T_4 is still of importance due to their biological and environmental persistence, investigating the impacts of alternative PFAS compounds will become more critical as human exposure to these compounds increases. The experimentation used in our analysis of PFOA and PFOS could be used to demonstrate the impact of alternative PFAS compounds on T_4 uptake. Gathering a larger library of PFAS impacts on T_4 uptake could help identify which PFAS compounds are suitable to use in products for human consumption.

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