# University of Alberta

Perfluorinated Acids in Human Serum as Determinants of Maternal Hypothyroxinemia

by

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## Dedications

This work is foremost dedicated to my family for their support throughout the years.

## Abstract

Perfluorinated acids (PFAs) are widespread global and human blood organohalogen contaminants. These monomer decomposition products used in surface treatment products and in fluoropolymer manufacturing and fire fighting may disrupt maternal thyroid hormone homeostasis given that animal studies demonstrate an apparent hypothyroxinemic condition upon PFA exposure. Firstly, we developed a method for properly quantifying perfluorohexane sulfonate (PFHxS), a PFA suspected of overreporting in past literature. We then investigated whether perfluorooctanoate (PFOA), PFHxS and perfluorooctane sulfonate (PFOS) were determinants of maternal hypothyroxinemia in a pregnant women population from Edmonton using a case-control design. Free thyroxine (fT4) and thyroid stimulating hormone (TSH) were screened in 974 women collected during 15-20 weeks of pregnancy. Cases (n=96, hypothyroxinemic: normal TSH and fT4: lowest 10th percentile) and controls (n=175, fT4: 50th and 90th percentile) were matched based on age and physician. Conditional logistic regression indicated that these PFAs are not associated with maternal hypothyroxinemia.

## Acknowledgements

I would like to acknowledge my supervisor and committee members for their support. I would also like to acknowledge my old friends who provided plenty of encouragement, as well as the new ones I've met along the way.

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## List of Abbreviations

| | |
|---|---|
| IPE | Ion-pair extraction |
| LC | Liquid chromatography |
| MS | Mass spectrometry |
| *m/z* | Mass to charge ratio |
| PFHxS | Perfluorohexane sulfonate |
| PFOA | Perfluorooctanoate |
| PFOSA | Perfluorooctane sulfonylamide |
| PFOS | Perfluorooctane sulfonate |
| SPE | Solid phase extraction |
| T3 | Triiodothyronine |
| T4 | Thyroxine |
| TSH | Thyroid stimulating hormone |
| TTR | Transthyretin |
| TRH | Thyrotropin releasing hormone |

# CHAPTER 1. Introduction: Overview of Perfluorinated Acids

## 1. 1. What are perfluorinated acids?

Perfluorinated acids are a family of chemicals that consist of a perfluorinated (i.e. every hydrogen has been replaced by fluorine) carbon backbone, generally 4-14 carbons in length, and a charged functional moiety that is usually a carboxylate, sulfonate or phosphonate.[1] These chemicals have been in use for the past half century.[1] They are stable at temperatures in excess of 150°C, are not readily degraded by oxidizing agents, alkalis or strong acids, and are nonflammable,[1] which makes them practically nonbiodegradable; thus perfluorinated acids are environmentally persistent.[2-4] Only advanced remediation techniques that incorporate a catalyst are capable of degrading perfluorinated acids, such as by irradiation, and persulfate or zero-valent iron in subcritical water. [5]

Perfluorinated acids are man-made chemicals and are generally produced by one of two common techniques: i) Simons Electrochemical Fluorination, and by ii) tetrafluoroethylene unit telomerization. The first process utilizes the reaction of an organic feedstock (1-octanesulfonyl fluoride ($C_8H_{17}SO_2F$) for perfluorooctane sulfonate (PFOS, $C_8F_{17}SO_3^-$) , and1-heptanecarbonyl fluoride ($C_7H_{15}COF$) for perfluorooctanoic acid (PFOA, $C_7F_{15}CO_2^-$), and anhydrous hydrogen fluoride fueled by an electrical current.[1] The current causes fluorine atoms to replace hydrogen atoms on the carbon backbone and results in a “perfluorinated” acid fluoride product such as perfluorooctane sulfonyl fluoride (POSF, $C_8F_{17}SO_2F$),[1]

which can then be used to synthesize PFOS, or various higher molecular weight substances. Various chain length impurities, as well as linear, branched or cyclic isomers result from the fragmentation and rearrangement of the carbon skeleton during electrochemical fluorination.[1] The second process, telomerization, reacts a molecule called a "telogen" with "taxogens" into a fluorinated telomer halide.[6] For example, the Du Pont Company developed a process involving reacting tetrafluoroethylene ($C_2F_4$) with iodide pentafluoride ($IF_5$) and iodine ($I_2$) to create the telogen, pentafluoroethyl iodide ($CF_3CF_2I$). [6] Even carbon numbered products are always resultant when both the telogen and taxogen contain an even number of carbons, and longer carbon chain lengths are yielded with the subsequent telomerization of tetrafluoroethylene and pentafluoroethyl iodide.[6] Afterward, even numbered straight-chain alcohols ($F(CF_2CF_2)_nCH_2CH_2OH$) are produced from the iodide, and these can be converted to various desired products.[7] Today, telomerization is currently the most common manufacturing route for perfluoroalkyl substances with straight chains of 6-14 carbons.[7]

The perfluorinated carbon chain can be credited for perfluorinated acids' low surface tension and their hydrophobic and oleophobic (literally oil-hating) nature.[1] Combined with the water soluble charged moiety, these properties make perfluorinated acids ideal surfactants.[1] The eight-carbon chemicals are the most effective surfactants, hence their high historical production volumes. POSF derived chemicals and telomer-related products are used in excess of 200 consumer and industrial applications including water-, stain-, and soil-resistant

coatings for clothing, leather, upholstery, and carpets, oil-resistant coatings for paper products for food, electroplating, electronic etching bath surfactants, photographic emulsifiers, aviation hydraulic fluids, fire-fighting foams, paints, adhesives, waxes and polishes, etc….[8] Fluoropolymers such as polytetrafluoroethylene (e.g. Teflon), and fluoroelastomers require perfluoroctanoic acid (PFOA) as an emulsifier during their production.[1]

## 1. 2. Analytical Chemistry

The first clues of widespread perfluorinated acid occurrence in humans were revealed by Taves *et al.* in 1968.[9] Using nuclear magnetic resonance (NMR), no authentic standards, and battling low concentrations, the presence of PFOA or related substances in human blood was strongly suggested, but no concrete identification of the specific perfluorinated acids could be made at the time. Eventually, owing to new technology and analytical methods involving the linking of high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS), the first reports that unambiguously demonstrated that humans and wildlife had perfluorinated acids in their blood and tissues (particularly liver) were made.[10, 11] This new method allowed ng/mL or ng/g (part-per-billion) concentrations to be measured with relative ease using clever extraction techniques based on ion-pair extraction into organic solvent, and requiring only small volumes, or mass, of sample owing to tandem MS which is both highly sensitive and selective (i.e. mass specific).

In 2001 two landmark publications introduced the world to the extent of perfluorinated acids' occurrence in the environment and in humans.[10, 11] Giesy *et al.*were the first to report on the global distribution of perfluorooctane sulfonate (PFOS) in wildlife, while Hansen *et al.* were the first to report on perfluorinated acid contamination in recent samples of human sera. Giesy *et al.*measured PFCs in wildlife tissues including fish, birds, and marine mammals from urbanized areas in North America, namely the Great Lakes region, coastal marine areas and rivers as well as in Europe, and also in remote areas such as the Arctic and North Pacific Oceans. PFOS was found to be widespread in the environment. Comparing concentrations in the same species living in industrialized regions (North American Great Lakes, Baltic Sea, and Mediterranean Sea) to those in more remote regions clearly showed that greater PFOS concentrations were present in animals living closer to human activity. Additionally, predatory animals such as bald eagles and mink had PFOS concentrations that were greater than in their respective diets, thus suggesting PFOS' bioaccumulation potential to higher trophic levels in the food chain. Mink from the Midwestern USA had the greatest mean concentrations of all species examined (mean: 2630 ng/g, range: 970-3680 ng/g wet weight). Giesy *et al.* also looked for three other fluorinated organic compounds including perfluorooctane sulfonylamide (PFOSA, $C_8F_{17}SO_2NH_2$), perfluorohexane sulfonate (PFHxS, $C_6F_{13}SO_3^-$) and PFOA. These chemicals were often detectable but not at concentrations as high as PFOS. PFHxS concentrations were below the limits of quantification (LOQ, ranged from 1 to 86 ng/g wet wt)

and a few samples contained PFOA or PFOSA at levels greater than LOQ of 2.5-180 ng/g and 1-38 ng/g wet wt respectively.

Hansen et al. reported PFOA and PFOS detection in all human sera samples (obtained from a private laboratory, n=65), though PFOS was reported at much higher average concentrations (± standard deviation) than PFOA (28.4 ± 13.6 ng/mL and 6.4 ± 4.8 ng/mL, respectively). Unlike Giesy et al.'s wildlife study, PFHxS concentrations (6.6 ± 5.1 ng/mL) were slightly higher than PFOA in humans, although not likely statistically different.

Perfluorinated acids are now recognized to be ubiquitous in humans. National Health and Nutrition Examination (NHANES) studies utilize serum samples collected from the United States population, with representation from both sexes, three races/ethnicities and four age categories per year. PFOA, PFOS and PFHxS were found in all samples during the NHANES 1999-2000 (n=1562) and 2000-2001 (n=1832) studies, confirming widespread perfluorinated acids exposure.[12, 13] The NHANES 2003-2004 study (n=2094) detected PFOS, PFOA and PFHxS in more than 98% (n=2094 participants) of the samples with PFOS concentrations being generally highest, followed by PFOA and PFHxS.[13] This study showed a significantly lower geometric mean concentration (approximately 25% for PFOA, 32% for PFOS, and 10% for PFHxS) than those found in the 1999-2000 study ($p<0.001$). These observations may be attributed to the discontinuation of electrochemical fluorination production by the 3M Co., beginning in 2000.

A unique observation in these NHANES studies includes racial differences that were reported in all years. Mexican-Americans displayed the lowest geometric mean concentrations of perfluorinated acids in the 1999-2000 study versus the 2003-2004 reports.[13] For PFOA, all racial differences were statistically significant, at all age groups. The 2001-2002 study showed the highest concentrations of PFOA, PFOS and PFHxS from non-hispanic white males.[14] Interestingly, non-Hispanic whites had PFC concentrations that were approximately two fold greater than those of non-hispanic blacks and 3 fold greater than Mexican Americans. Racial differences were age dependent for PFOS, but sex dependent for PFOA.[14] Lifestyle, genetic variability, diet or a combination of these factors may account for these differences seen amongst groups.[14]

Age, in contrast to sex, does not seem to strongly influence PFA concentrations, albeit few measurements are available for infants and very young children. In the 2001-2002 study, PFA concentrations were similar between all age groups, whereas adult males (PFOS: 37.8 ng/mL, PFOA: 4.9 ng/mL) had higher geometric mean concentrations than females (PFOS: 32.1 ng/mL, PFOA: 4.2 ng/mL).[14] Concentrations of most PFAs were higher in males versus females but similar between all age groups, with the exception of PFHxS in the NHANES 1999-2000 study.[15] Here, PFHxS concentrations were higher in children (12-19 years old) and adolescents (20-39 years old) than in the older populations. Calafat et al. proposed that the younger groups may have had more contact with carpeted

floors which may have been treated with postmarket carpet-treatment applications containing PFHxS.

Despite the inherent specificity of HPLC-MS/MS to monitor for perfluorinated acids, the method is not fool-proof and care must be taken to maintain quantitative accuracy. For example, a common disadvantage of electrospray ionization, the technology used to link HPLC with MS, is a potential bias known as "ionization suppression" or "ionization enhancement" which may be caused by various sample matrixes. Such effects may be caused by ionization competition between different species eluting simultaneously, or even by coeluting analytes.[16, 17] Isotopically labeled internal standards (e.g. $^{18}O$, or $^{13}C$) are the best approach to correcting for any matrix effects by mass spectrometry, but these have only recently become available thus some of the oldest perfluorinated acid data may suffer from inaccuracy. Using the isotopically labeled internal standard, which has the same retention time as the corresponding native analyte, and correcting the response of the analyte for the response of its respective internal standard negates the matrix effect. Care must still be taken to "clean up" the sample before injection to HPLC-MS/MS to minimize the matrix effects because sensitivity is also drastically reduced through ionization suppression.

Another early problem in perfluorinated acid analysis was that early authentic standards contained many impurities, for example with PFOS purity ranging from

86-98%. Low purity standards may result in a positive bias when used for quantification. However, today most authentic standards are highly pure.

The presence of multiple branched and linear perfluorinated isomers may also create another problematic situation. For example, eleven PFOS isomers found in standards have been matched to those detected in human sera.[18] Most routine methods still ignore these isomers and they are eluted simultaneously by HPLC, but because each of these has its own ionization and MS/MS characteristics,[18] a systematic bias may result unless the relative amounts of branched isomers in the authentic standard are identical to those of the sample.

Another potential problem arises with mass interference from small biomolecules. PFOS [10, 18] and PFHxS [18] were reported to have interferences in some mass transitions when biological samples are analyzed. Enhanced product ion scans identified an interferent for PFOS as isomers of taurodeoxycholate[18], bile acids which co-elute with the perfluorinated acids as a result of similar physical properties.[18] Additionally, a recent study reported that two endogenous steroid sulfates caused a 10 to 20-fold overreporting for PFHxS in pooled maternal serum.[18] Furthermore, previous studies have indicated difficulties in collecting data for PFHxS. For example, Apelberg *et al.* did not report PFHxS concentrations in cord blood sera due to these interferences,[19] and others have reported that they combined the area counts for PFHxS with the potential interferents,[20] possibly thinking that these were isomers of PFHxS. Until now, the

extent of this systematic bias for PFHxS was unknown for individual maternal sera, men or non-pregnant women; this will be examined in Chapter 2.

## 1. 3. Toxicity of Perfluorinated Acids in Animal Studies

Toxicity studies on animals have focused mostly on PFOS with some studies on PFOA and only one for PFHxS. Here we will mainly report on the adsorption, distribution, metabolism, excretion (ADME) and toxicity for PFOS, and when information is available, for PFOA and PFHxS. Studies on other longer or shorter perfluorinated acids will be reviewed when relevant.

### 1. 3. 1. ADME of Perfluorinated Acids

Animal studies show that both PFOA and PFOS are well absorbed orally.[21] For example, this was evidenced by >95% oral absorption in toxicokinetic studies with radiolabeled PFOS ($^{14}$C) in male rats. [21] The same study showed the radiolabeled PFOS distributed mostly to the liver and serum, with evidence of enterohepatic circulation.[21] After 89 days, mean tissue concentrations (μg/g) of $^{14}$C-PFOS were: liver 20.6, plasma 2.2, kidney 1.1, lung 1.1, spleen 0.5, and bone marrow 0.5. The adrenals, skin, testes, muscle, fat and eye contained concentrations <0.5, while no radioactivity was found in the brain.[21] This is consistent with Harada *et al.* who were able to demonstrate that PFOA and PFOS

cannot readily pass through the blood-brain barrier in humans.[22] This was evidenced by lower ratios of PFOS in cerebrospinal fluid versus the serum. In contrast, a higher transport into bile of PFOS and PFOA were shown.[22] Finally, PFOS and PFOA have been reported in umbilical cord blood serum, which provides evidence that these PFCs can cross the placenta.[23, 24]

Both PFOS and PFOA are not metabolized, as indicated by animal studies.[21] However, rat and fish studies have indicated that "precursor compounds" are capable of producing PFOS and other perfluorinated acids through metabolism.[7] For example, various substituted perfluorooctanesulfonamides ($C_8F_{17}SO_2NR^1R^2$) have been found in animals, humans and the environment, but usually at lower concentrations than PFOS. Subacute studies in female rats exposed to N-ethyl perfluorooctanesulfonamidoethanol (N-EtFOSE) indicated that metabolism of these chemicals can yield PFOS. (Figure 1) [25]

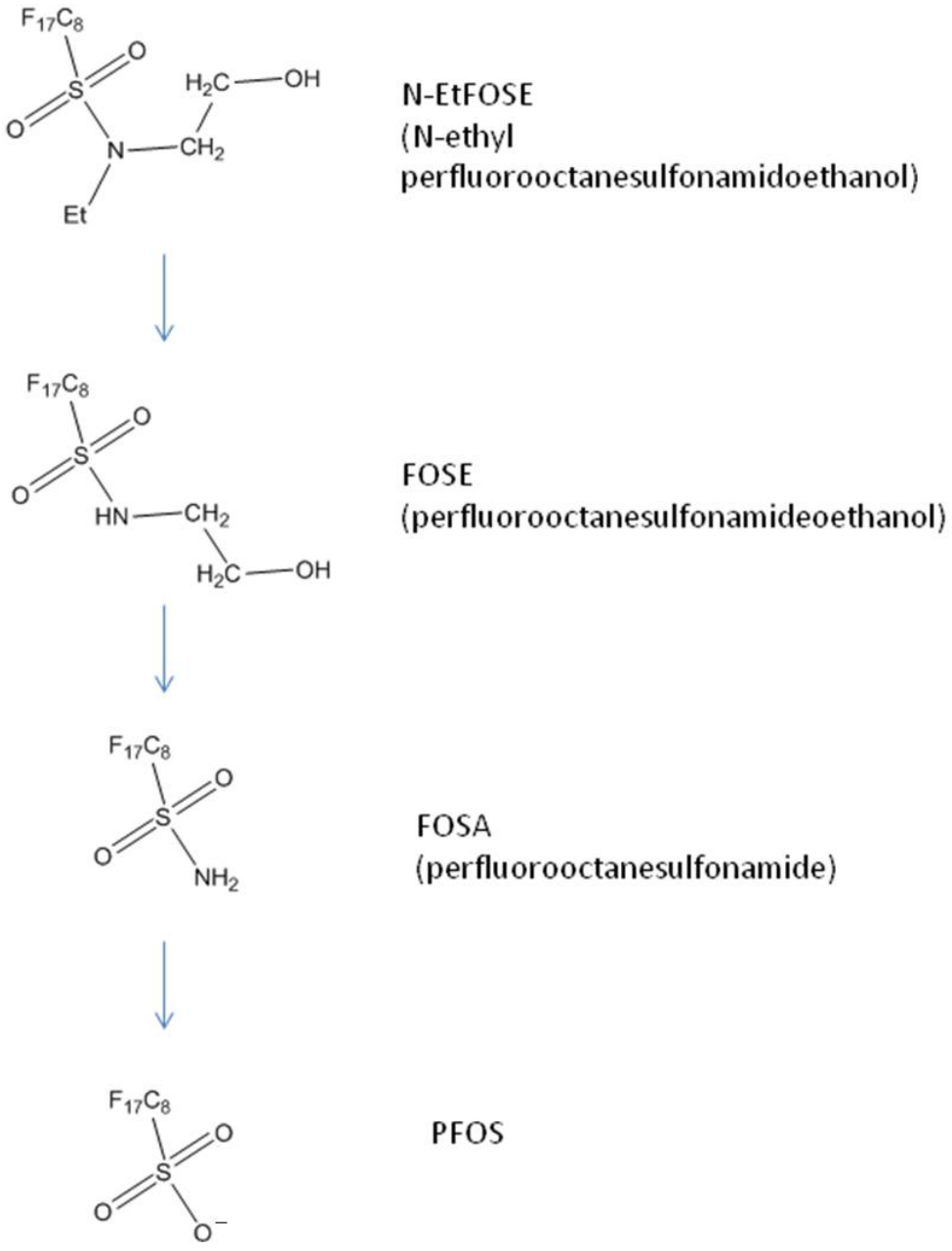


**Figure 1-1. Biotransformation pathway of N-EtFOSE to PFOS.**

Other precursor compounds, produced by telomerization, can form PFOA. It has been hypothesized that fluorotelomer alcohols (FTOHs; CF3(CF2)*x*C2H4OH;

where $x$ = 3, 5, 7, 9), which are found in the North American atmosphere, may be possible precursors for perfluorinated acids in human blood.[26] Through examining metabolic products produced by isolated rat hepatocytes with 8:2 FTOH, it was determined that the fluorotelomer alcohol could form PFOA with a telomer aldehyde as an intermediate.[26]

Generally, the rate of elimination of perfluorinated compounds is increased with decreasing chain length. However, the exception may be for PFHxS, compared to PFOS, in humans.[1] The half life of PFHxS has been demonstrated to be greater than PFOS, as seen in retired fluorochemical production workers.[28] Also, half lives differ greatly by gender and species. For example, the elimination half life of PFOA in adult male rats is 4-6 days, whereas for females it was 2-4 hours.[29] Here, gender specific expression of organic anion transporters in the kidney may explain these results.[30] Furthermore, in some species sex steroids also appear important since PFOA elimination was slowed by testosterone administration to female and castrated male rats [30] and was increased in males receiving estradiol.[31] Similarly, female dogs demonstrated a PFOA half life of 8-13 days versus male dogs with 20-30 days.[32] In contrast, female cynomolgus monkeys showed a half life of 30 days for PFOA while males had a half life of 21 days.[33] Additionally, female hamsters did not excrete PFOA faster than their male counterparts [32] and mice and rabbits also did not show any differences in elimination half lives.[34]

In humans, differences in elimination half lives between males and females have varied. Olsen *et al.* found that the one female subject in their study had an elimination half life that was twice that of the males (n=25): 45.7 days versus 24.1 days for perfluorobutane sulfonate (PFBS, $C_4F_9SO_3$) respectively.[35] A study on a Kyoto population showed that females over 51 years old (n= 5, all menopausal), had significantly higher serum concentrations of PFOA and PFOS than females in the 20–50-year-old age group (n= 5, actively menstrual): mean and SD PFOA (7.89 ± 3.61 ng/mL and 12.63 ± 2.42 ng/mL for younger and older women), PFOS (13.18 ± 5.03 ng/mL and 24.00 ± 7.55 ng/mL for younger and older women).[36] Harada *et al.* suggested that menstrual bleeding might be the sex specific route of elimination in females.[36]

Humans have a much longer pharmacokinetic half-life for perfluorinated acids (PFOA: 3.5 years, PFHxS: 7.3 years, PFOS: 4.8 years) [28] than other species. The reasons for this are not clear. Anderson *et al.* proposed enhanced reabsorption processes as the mechanism for longer half lives of these chemicals in humans.[37] Predicted resorption rates of PFOA and PFOS from rats were estimated to be 0.89 and 0.97 respectively (where 1.0 is complete resorption),[22] and were similar to those found in male rats dosed orally with PFOA and PFOS (0.93 and 0.95).[38, 39] Harada *et al.* speculated that these high resorption rates are needed for enterohepatic circulation and may result in the long half lives found in humans.[22] A transporter protein may play a role in the biliary excretion of PFOA and PFOS, but there has not been any research to date that have studied these [22], thus

differences in species-species expression of such proteins cannot be discussed. More studies are needed to show which exact transporters are used to transfer perfluorinated acids into tissues and out of the body.

## 1. 3. 2. General Toxicities of PFOA, PFOS and PFHxS

Perfluorinated acids have demonstrated a variety of toxic effects in acute and/or subchronic exposure animal studies: total serum cholesterol reduction,[40, 41] hepatic peroxisome proliferation ,[42-46] intracellular gap junction communication disruption,[47, 48] and hepatotoxicity [40, 41, 45, 49, 50] are a few most notable and reproducible effects. Peroxisome proliferation is commonly seen in animal studies and results vary between species: rat peroxisome- activated receptor alpha (PPAR-α) was demonstrated to be least responsive to PFOA and PFOS while human PPAR-α was the most responsive.[51] Peroxisome proliferation as well as interference of gap junction homeostasis has been suggested to be related to general hepatotoxicity and hepatocarcinogenicity, but these cause-effect relationships are inconclusive. Experiments have shown serum cholesterol and triacylglycerol reductions in rats (administered 20 ppm dietary PFOS for 14 weeks),[40] and in rhesus and cynomolgus monkeys[41] (at 1.5 mg/kg/d dose groups for 90 days and 0.75 mg/kg/d dose for 182 days, respectively). The only study on PFHxS effects was for male and female rats (n=15 rats per treatment group) dosed with 0.3, 1.3, and 10 mg/kg day, and there were differences between the two sexes.[52] Although females did not show any effects, males dosed with PFHxS for

42 days exhibited similar responses to PFOS and PFOA, with decreased cholesterol levels at all doses, increased liver to body weight ratio, increased liver to brain weight ratios at 3 and 10 mg/kg-d, and decreased triglycerides at 10 mg/kg/d.[52] This study also showed male rats had decreased hematocrit (at 3 and 10 mg/kg/d) and increased albumin concentrations at 10 mg/kg/d.[52]

### 1. 3. 3. Reproductive and Developmental Animal Toxicity of PFOA and PFOS

Reproductive toxicology examines the adverse effects of substances on the reproductive system of the parent generation which may diminish the capacity for successful fertilization. Developmental toxicology, on the other hand, focuses on post-fertlization effects spanning from the embryonic period to the fetal period [53], and viability and teratogenicity are common endpoints examined. Here, we will examine the reproductive and developmental toxicities of PFOA and PFOS in animal models. Butenhoff *et al.* is the only reproductive and developmental study for PFHxS, and here female rats were dosed and subsequently the dams and their offspring did not show any treatment-related effects.[52] Thus under these conditions, it was concluded that PFHxS was not a developmental or reproductive toxicant; albeit this is the only study conducted to date, thus further animal investigations with PFHxS are warranted in independent laboratories.

### 1. 3. 4. Birth Outcomes

#### 1. 3. 4. 1. Teratogenicity

When exposed *in utero* to PFOS, rat and mice offspring displayed various birth defects such as delayed ossification (sternebrae, phalanges), craniofacial malformations (cleft palate), anasarca, and cardiac defects (right atrium enlargement, ventricular septal defects), particularly at 10 and 20 mg/kg PFOS (dams dosed daily from gestational day 2 to 20 for rats and gestational day 1 to 17 for mice).[54]Other developmental delays included growth retardation (such as body weight gain disruptions at 2 mg/kg) and delays in eye opening in rats and mice.[55] Here, maternal weight gains in both mice and rats were decreased by PFOS in a dose-dependent manner, which Thiobodeaux *et al.* suggested may have occurred from suppressed food and water intake.[54] The high-dose group dams had significantly reduced serum triglycerides with no differences in cholesterol concentrations. Consequently, these toxic effects on the mothers make the teratogenicity of PFOS rather ambiguous.

#### 1. 3. 4. 2. Viability

Increased mortality of offspring is often a final endpoint that is measured in reproductive studies and fetal viability is a varied outcome. In the aforementioned study, Thibodeaux found little adverse effects on numbers of implantations or live fetuses in PFOS exposed rats and mice.[54] However, in a follow up study described by Lau *et al.*, of the postnatal period in both rodents, the low dosage of PFOS (2

mg/kg) increased infant mortality.[55] The PFOS body burden and resulting morbidity and mortality of newborn rats were directly related. Although all rats were born alive from all dosage groups, survival was only a few hours at the highest dosages (5 and 10 mg/kg).[55]

The mechanism of neonatal death from PFOS is not fully understood. It was suggested that organ systems developing in the late stages of gestation are more vulnerable to PFOS, as pregnant rats administered PFOS during the last days of gestation showed a comparable pattern of neonatal death,[57] although concentrations used in the Grasty *et al.* study [57] were higher than the Lau *et al.* reports. [41] Lau *et al.* further suggested that the likely mechanism for mortality was interference with lung and pulmonary function [58] because the effects were similar to those seen with nitrofen, a herbicide that suppresses fetal lung maturation leading to cardiopulmonary function disruption, and ultimately death in newborn rats.[59-61] Comparatively, enlarged right atriums in PFOS exposed fetuses found in the Thiobodeaux study may have occurred as a result of pulmonary hypertension.[54]

Another possible mechanism for increased perinatal mortality includes disruption of glycogen store homeostasis. PFOS treated dams (1.6 mg/kg/d) showed an apparent increase in glycogen stores in their day old rat pups, visualized through electron microscopy of liver cells.[62] In early postnatal periods, large amounts of hepatic glycogen accumulates in fetal liver at the end of gestation, where these

stores are moved as free glucose to maintain newborn blood glucose levels and for life sustainment until suckling and gluconeogenesis onset.[63, 64] Increased perinatal mortality can occur with a decrease in glucose delivery to the developing offspring.[65] Luebker *et al.* proposed that PFOS may prevent glycogen store utilization, but this hypothesis was not supported by their data, which suggested that glycogen mobilization was not inhibited since liver glycogen and serum glucose concentrations were not significantly decreased (0-2.0 mg/kg day for 42 days).[66]

## 1. 4. Thyroid Hormone

### 1. 4. 1. What is thyroid hormone?

The human thyroid gland is found at the base of the neck and around the trachea. It is composed of about three million follicles, or epithelial cells, arranged in hollow vesicles filled with thyroglobulin (a glycoprotein colloid).[67] Aside from homeostasis, thyroid hormone produced from the thyroid gland helps maintain normal blood pressure, metabolic rate, heart rate, muscle tone, cardiac performance, body temperature, digestion and reproductive functions.[67] In the developing fetus, thyroid hormone is responsible for terminal brain differentiation regulation, as well as synaptogenesis, neuronal migration and myelination.[68, 69] Additionally, thyroid hormone is responsible for the normal development of inner ears, lungs and nervous systems (mostly the central nervous system). [67]

Thyroid hormones have a high affinity for serum proteins, but it is the free serum concentrations of thyroid hormone are critical to their function, and this is regulated by negative feedback through the hypothalamus-pituitary gland-thyroid gland (HPT) axis. (Figure 2) In response to low circulating thyroid hormone concentrations, the hypothalamus releases thyrotropin releasing hormone (TRH). This is sensed by the anterior pituitary, which increases expression of thyroid stimulating hormone (TSH). Together, TRH and the anterior pituitary gland control the upregulation for thyroid hormone secretion. TSH is released into circulation and can bind to the TSH receptors in the thyroid gland which triggers a signal transduction pathway, leading to the release of T3 and T4 from the gland into the bloodstream in a 1:20 ratio, respectively.[67]

When circulating thyroid hormone concentrations are sufficiently high, negative feedback inhibition occurs through thyroid hormone action on TRH neurons in the paraventricular nuclei of the hypothalamus and on thyrotropes (cells of the anterior pituitary). Animal studies indicate that T3 and T4 inhibit TRH synthesis and secretion. Normally, T4 and T3 enter the cell (only the free/unbound hormone fraction can cross the cell membrane), where T4 can be deiodinated to T3 in the cytoplasm. When these hormones are in excess, T3 can enter the nucleus and bind to specific receptors that down-regulate the gene transcription of TSH and TRH receptors. T3 also inhibits stored hormone release and increases TRH receptor degradation. The final result is a decrease in thyrotrope sensitivity to TRH.[67] (Figure 1-2)

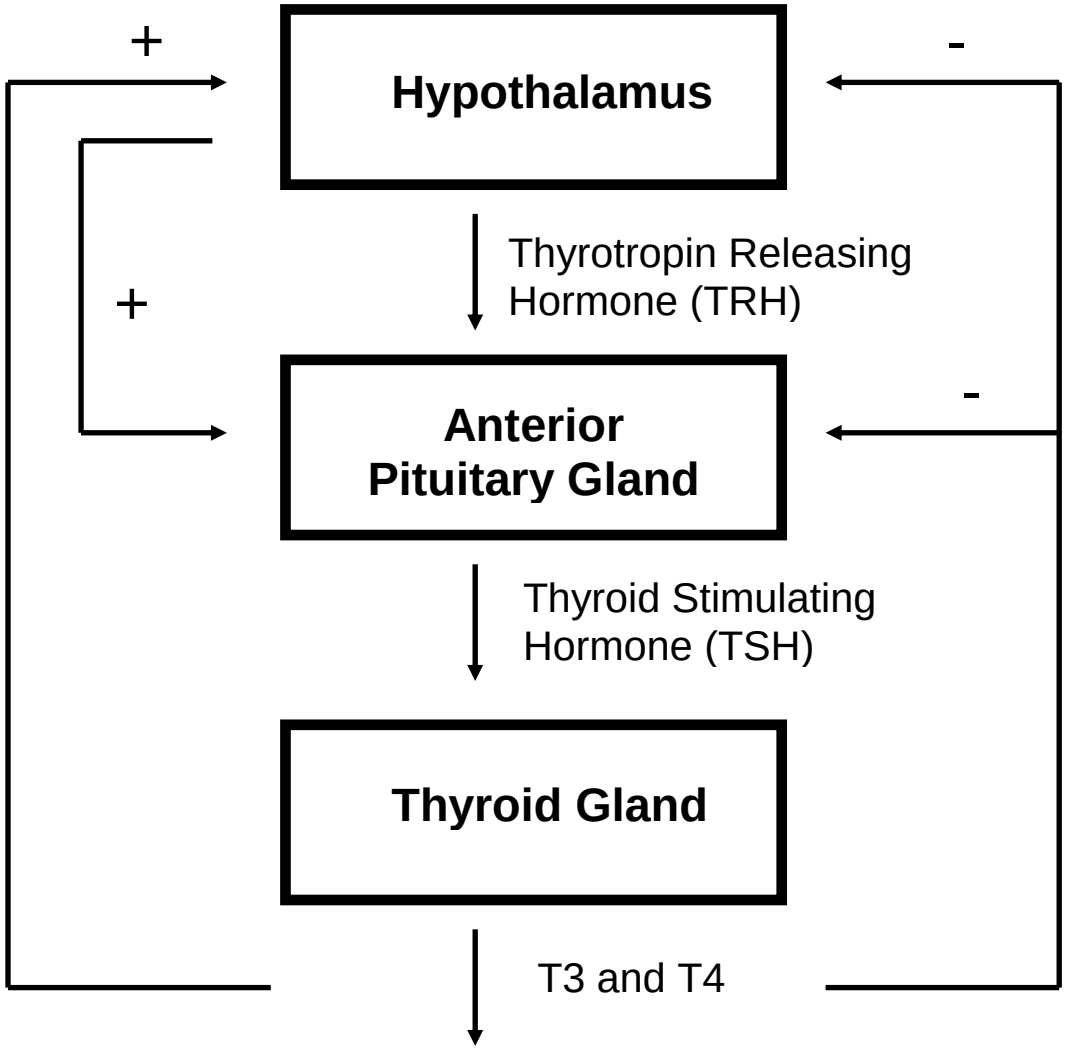


**Figure 1-2 Negative and positive feedback pathways of the thyroid hormone system**

Thyroid hormone synthesis takes place in the thyroid follicular cell. Iodide (I-) is transported into the follicular cell through the sodium iodide symporter (NIS) and into the lumen through an iodide channel (pendrin) (Figure 1-3). Thyroglobulin

(TG) is produced in the nucleus and released into the lumen. In the presence of hydrogen peroxide, thyroid peroxidase (TPO) reacts oxidized iodide with tyrosine residues in TG to yield monoiodotyrosol (MIT) and diiodotyrosol (DIT). Coupling of one MIT and one DIT yields T3, whereas the coupling of two DITs yields T4 (structures of T3 and T4 are shown in Figure 1-4). Release of T3 and T4 into the blood occurs through phagocytosis.

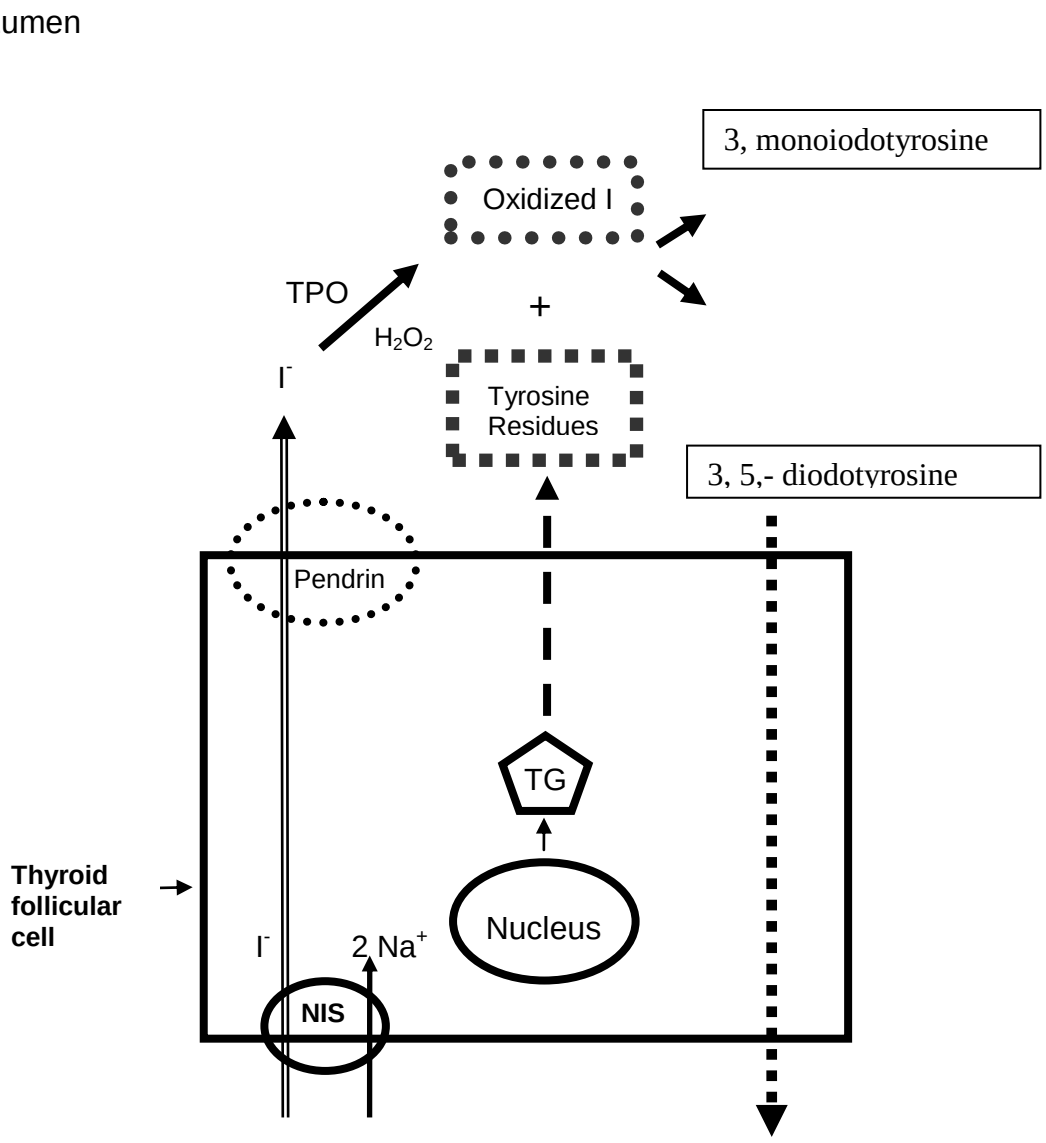


**Figure 1-3 Thyroid hormone precursor biosynthesis.**

Three deiodinase enzymes are responsible for T4 metabolism. (Figure 1-4) Type I deiodinase is located primarily in the liver and kidney, but also in the central nervous system, the anterior pituitary gland, and the thyroid gland. This deiodinase enzyme removes an iodine atom from the outer (phenolic) ring or from the inner (tyrosyl) ring. Iodine atoms located in the phenolic ring are referred to as 3' and 5', whereas those in the inner ring are known as 3 and 5. Removal of an iodine from the outer ring produces T3, or 3', 3, 5 triiodothyronine. Iodine removed from the inner ring of T4 produces reverse T3 (rT3), which cannot bind to the thyroid hormone receptor and is further deiodinated. Type II deiodinase is found primarily in the brain and the pituitary gland, where the production of T3 from T4 is used to meet local tissue demands independent of circulating T3 concentrations. Type II deiodinase expression is governed by other hormones, and expression is highest when blood T4 concentrations are low. Type III deiodinase's primary purpose is for degradation and removes an iodine from the tyrosol ring of T3 or T4. It is found in many tissues, where rT3 is generated from Type I and Type III enzymes. Further deiodination occurs through rT3 by removal of a second iodide from the inner ring. Reverse T3 is degraded faster than T3, while some rT3 can escape into the bloodstream, where it can be bound to proteins including its carrier proteins: thyroid binding globulin (TBG) or transthyretin (TTR). [67]

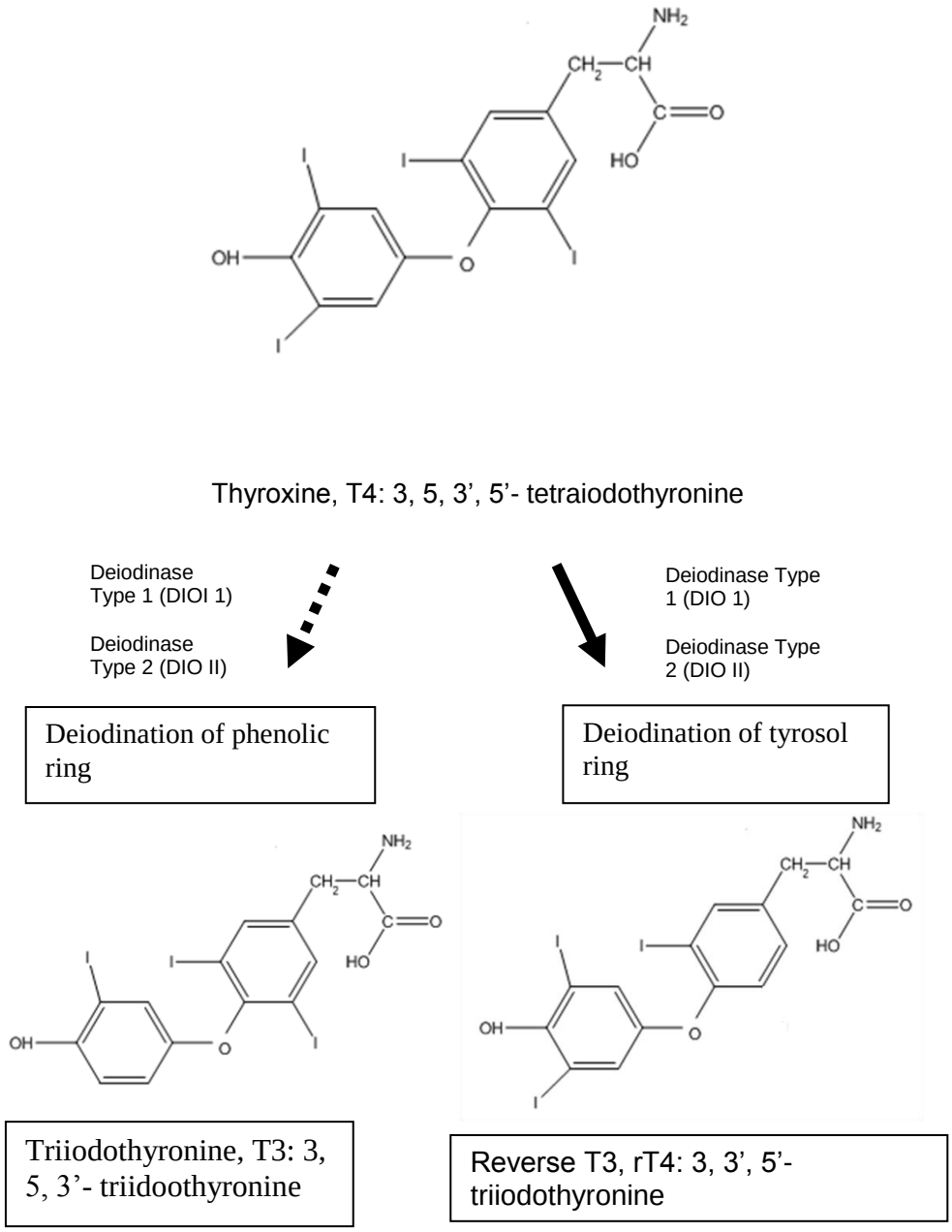


**Figure 1-4 Metabolism of thyroxine.**

Thyroid hormone transport in the serum occurs mainly through binding to three proteins: TBG, TTR, and albumin. More specifically, one binding site for T3 or

T4 is available for each TBG protein, and in humans approximately 70% of T3 and T4 are transported through this protein as a result of their high affinity for TBG.[70] Concentrations of TBG in the blood are usually 280-560 nmol/L.[70] TTR has a tenfold greater relative affinity for T4 compared to T3, and in humans this protein transports about 10% of thyroid hormone around the body.[70] Albumin binds to T3 and T4 with the least affinity, but due to its high plasma concentrations albumin accounts for 15% of total thyroid hormone transport.[70]

### 1. 4. 2. Importance of Thyroid Hormone During Early Pregnancy

Throughout pregnancy, thyroid hormone levels change drastically. During the first and second trimesters, maternal circulation contains greater free T4 concentrations than in fetal circulation.[71] Human fetal brain contains T3 receptors in the first trimester, and thyroid hormone deiodinases and receptor isoforms are already present in the human fetal cerebral cortex at the midpoint of the first trimester of pregnancy. It has been postulated that the fetal CNS has the capacity to respond to T3 that may be created locally or transported transplacentally.[72] This demonstrates the importance of maternal free T4 for adequate fetal T3. T4 itself is detectable in the fetal brain at 11-14 weeks (first trimester), and increases about 2.5 times by 15-18 weeks.[72] Moreover, the fetus depends on transplacental transfer for T4 and iodine supply.

The placenta and fetus contain high levels of deoiodinase III and sulfo-transferase activity, which can inactivate excess free T3 or free T4 that escape the placental

barrier into the fetus. These actions account for the low free T3 concentrations found in the early fetus. Placental deoiodinase III are not affected by maternal concentrations of free T4. This was evidenced by positive correlations between T4 in fetal fluids and circulating T4 or free T4 levels in their euthyroid mothers. The placenta is permeable to iodine and TRH, but not to TSH. Maternal TRH may be responsible for fetal thyroid function before fetal HPT axis maturation, which does not happen until near the end of term.[73]

The fetus eventually begins to produce its own thyroid hormone during the second trimester, but maternal thyroid hormone is still significant in its supply to the fetal brain. Generally, concentrations of total T4, free T4, and free T3 increase in the fetus during gestation.[74, 75] Maternal T3 concentrations are two to three times higher than those levels in the fetus at birth, and at this stage about 30% of thyroid hormone in cord blood is from the mother.[71] During this time, the placenta also degrades much of the free T4, but some is still transferred.[76] Additionally, thyroid hormone receptor (TR) binding capacity and T3 binding capacity in the brain increase throughout gestation, where a tenfold increase in the maximal binding capacity for T3 in human fetal brains occurs between 10 and 16 weeks.[75]

### 1. 4. 3. Animal Studies of PFOA and PFOS and Thyroid Effects

Decreases in circulating hormones, mainly thyroxine, are a commonly measured adverse effect of perfluorinated acids. (Table 1) The earliest reports demonstrating disruption of thyroid hormone homeostasis by a perfluorinated acid were by Langely *et al.* and Gutshell *et al.* 25 years ago, whereby a single dose of perfluorodecanoic acid (PFDA, $C_9F_{19}COO^-$) caused significantly reduced serum T3 and T4 concentrations in rats.[77, 78] A similar effect has also been observed in many other studies amongst various animals such as cynomolgus monkeys, rats, and mice.[50, 54, 55] Curious however, is the general absence of any compensatory increase in TSH; most studies have demonstrated no change in TSH over time despite decreased free thyroid hormones. Absence of a compensatory TSH increase with a decrease of T3 and T4 concentrations is not a unique observation to perfluorinated acids, this has also been reported with polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers.[79]

**Table 1-1 Animal studies and thyroid hormone effects with PFOA and PFOS.**

| Study | Chemical | Species | Dose, Duration, Route of Exposure | Effect |
|---|---|---|---|---|
| Butenhoff *et al.*, 2002 | PFOA | Cynomolgus monkeys (males) | 0, 3, 10, 20/30 mg/kg/day for 26 weeks orally | fT4, TT4 and TSH were not significantly altered. fT3 and TT3 were significantly decreased in the highest dose (20/30 mg/kg/day). |

| | | | | |
|---|---|---|---|---|
| Seacat *et al..*, 2002 | PFOS (PFOS potassium salt) | Cynomolgus monkeys (males and females) | 0, 0.03, 0.15, 0.75 mg/kg/d for 182 days, orally | Lowered TT3, increase in TSH (almost twice the control value) without evidence of hypothyroidism. Effects seen only at the 0.75 mg/kg/d dose. |
| Thibodeaux *et al..*, 2003 | PFOS | Pregnant Sprague-Dawley rats and mice | Rats: 1, 2, 3, 5, 10 mg/kg/d orally (gavage) from GD 2 to 20<br><br>Mice: 1, 5, 10, 15, 20 mg/kg/d from GD 1 to GD 17 | Serum TT4, fT4 and TT3 in rat and mice dams were significantly reduced as early as 1 week of exposure but no feedback response of TSH. |
| Lau *et al..*, 2003 | PFOS | Newborns of rats and mice from previous Thibodeaux *et al.*study | Rats: 1, 2, 3, 5, 10 mg/kg/d orally (gavage) from GD 2 to 20<br><br>Mice: 1, 5, 10, 15, 20 mg/kg/d from GD 1 to GD 17 | Rat pups: TT4 and fT4 were suppressed with no changes in TT3 or TSH (as early as postnatal age of 2 days and persisted onto day 35)<br><br>Neonatal mice: TT4 levels in 5 and 10 mg/kg tended to be lower, but results were not as consistent as with rats (not significant) |
| Luebker *et* | PFOS | Sprague- | 0.0, 0.4, 0.8, | Dams: |

| | | | | |
|---|---|---|---|---|
| *al..*, 2005 | | Dawley rats | 1.0, 1.2, 1.6, and 2.0 mg/kg/d (dosing of dams occurred for 6 weeks prior to mating with untreated males, followed through mating, gestation, and day four of lactation) | Significant reductions for TT3 and TT4 (using RIA) with no changes in free T4 or TSH (using ED/RIA)<br><br>Pups: Significant reductions in fT4 and TT4 (down to immeasurable levels) with no change in free fT3 or TT3 in all treated groups. Follow up analyses showed significant reductions in TT3 and TT4 in 1.0 mg/kg/d (Advia) with no differences in fT3. No differences in fT4 (ED/RIA) between control and 0.4 mg/kg/d. TSH slightly elevated in 1.6 mg/kg/d in mothers, magnitude does not suggest hypothyroidism |
| Fuentes *et al..*, 2006 | PFOS | Pregnant female mice | 0, 1.5, 3, 6 mg/kg/d on gestation days 6-18 | Reductions in TT3, TT4, fT3 and fT4 of pregnant mice at term, but no |

| | | | | |
|---|---|---|---|---|
| | | | | significant differences between exposed and control groups |
| Martin *et al..*, 2007 | PFOA and PFOS | Male Sprague-Dawley rats | Single doses of 10 mg/kg/d (PFOS) and 20 mg/kg/d (PFOA) for one, three, or five consecutive days | Gene expression profiling of livers: perturbation of thyroid hormone metabolism genes<br><br>PFOA: significantly decreased fT4, TT4 and TT3 at all time points.<br><br>PFOS: significantly decreased fT4, TT4 at all time points. TT3 was decreased significantly only on day 5. |
| Chang *et al..*, 2008 | PFOS | A) Female Sprague-Dawley rats<br><br>B) Male Sprague-Dawley rats | A) Single oral dose of 15 mg potassium PFOS/kg body weight. Measurements taken at 2, 6, 24 hrs<br><br>B) PFOS (3 mg/kg-d) and/or/with 10µg/mL in water of | A) fT4 increased and TSH decreased at 6 hrs, and TSH returned to control levels by 24 hrs. TT4 decreased by 55% over 24 hrs. TT3 and rT3 decreased at 24 hrs but not as much as TT4. |

| | | | | |
|---|---|---|---|---|
| | | | propyl thiouracil (PTU). Hormones measured on days 1, 3, 7, and 8 | B) TSH not changed, while TT4 and TT3 decreased in male rats with PFOS. Pituitary response not changed with PFOS. |
| Yu *et al.*., 2008 | PFOS | Male Sprague-Dawley rats | 1.7, 5.0, 15.0 mg/L in drinking water for 91 days | TT4 decreased significantly in all dosage groups, TT3 increased only at 1.7 mg/L. No significant increases of TSH. |

## 1. 5. Hypothyroidism and Hypothyroxinemia

Hypothyroidism is a condition defined by an increase in TSH concentrations, irrespective of free T4 concentrations. However, most animal studies involving perfluorinated acid exposure and thyroid hormone examination show decreases in thyroid hormone with no increases in TSH concentrations, an effect that is consistent with the condition known as hypothyroxinemia. One exception is a study by Luebker *et al.*, whom demonstrated developmental delays in a two-generational study with PFOS despite a euthyroid state, as changes in thyroid hormone were within the reference range.[62]

Hypothyroxinemia is defined as relatively low free T4 concentration (i.e. often defined by percentile, such as the 10$^{th}$ centile) with TSH in the normal reference range of concentrations. Hypothyroxinemic outcomes in animals following perfluorinated acid exposure have been presented in a number of studies. For example, Thibodeaux *et al.* reported significantly reduced serum thyroxine concentrations in PFOS treated rat dams with no feedback response of TSH.[54] PFOS exposure induced significant decreases in total and free thyroxine serum levels at all dose concentrations (1, 2, 3, 5, 10 mg/kg daily by gavage) as early as gestational day 7. An additional study with non pregnant rats showed markedly reduced serum T4 (total and free) concentrations with PFOS exposure as early as 3 days after treatment. However, TSH response in this group was variable and was dose and time dependent: serum TSH was increased at the 3 mg/kg dosage group, but after 7 days it was no longer significantly different from controls. Moreover, TSH changes after 20 days were not different from controls.[54]

A follow up study of the rat pups from the above study reported suppressed thyroxine concentrations in PFOS treated rat pups at all maternal dose concentrations (1, 2, 3, 5, 10 mg/kg by gavage).[55] Again, no significant TSH alterations in comparison to controls were reported. In fact, significantly low free T4 and normal TSH (i.e. characteristic of hypothyroxinemia) was observed on the second postnatal day, the earliest period when hormonal levels can be measured reliably.[55] This effect on free T4 lasted the duration of the study (through to postnatal day 35). [55]

Likewise, a recent report by Yu *et al.* also demonstrated hypothyroxinemic-like conditions in male Sprague-Dawley rats exposed to 1.7, 5.0, and 15.0 mg/L of PFOS in drinking water for 91 consecutive days.[80] Here, there was a significant decrease in serum total T4 at all doses with no significant effect on serum TSH. These effects paralleled an upregulation of one isoform of liver uridine diphosphoglucuronosyl transferase, UGT 1A1, as well as an increase in thyroidal type 1 deiodinase (DIO 1) in a dose-dependant manner.[80] Other factors such as thyroid peroxidase, sodium-iodide symporter, and TSH receptor mRNA were unaffected.[80] Thus, it is possible that hepatic T4 glucuronidation was increased through UGT1A1 and that the hypothyroxinemic-like state in rats may have furthermore been caused by increased thyroidal conversion of T4 to T3 through DIO 1.[80]

The apparent ability of perfluorinated acids to induce a hypothyroxinemia-like state, and the known importance of an adequate supply of maternal T4 during early gestation, raises questions about the possible effects of perfluorinated acids on the developing brain. In pregnant rat dams eating a low iodine diet, to induce hypothyroxinemia, neuronal migration into the neocortex was affected in pups. This resulted in permanent changes in the cytoarchitecture of the somatosensory cortex and the hippocampus.[81] In another study, the same authors treated pregnant rats with the goitrogen, 2-mercapto-1-methyl-imidazole (i.e. chemical that interferes with iodine uptake by the thyroid), for 3 days at the start of

neocorticogenesis and found that the mild hypothyroxinemia (although loosely defined, and despite TSH measurements not being available) produced similar cytoarchitectural changes in the somatosensory cortex and the hippocampus.[82] Changes in rat brain development can be paralleled with events occurring during human brain development, albeit with different time points. For example, postnatal maturation during rat brain development corresponds to the final stages of intrauterine life in the human brain.[46] Similarly, the same study demonstrated that the first trimester of pregnancy is the critical time for free T4's effects on normal brain development.[83]

### 1.5.1. Possible Mechanisms for T3 or T4 Disruption by Perfluorinated Acids

The decrease in free T4 and T3, with or without the compensatory increase in TSH, has been widely studied in animal models. Plenty of general mechanisms have been proposed, and vary from increased thyroid hormone turnover with malic enzyme (involved in carbohydrate catabolism) to increased excretion through the urine.

PFOS has clearly been implicated in thyroid hormone homeostasis disruption in animal studies. An important clue as to the mechanism may come from Chang *et al.* who reported an increase in serum free T4 and a decrease in serum TSH over the first 6 hours in rats administered a single dose of PFOS.[84]However, free T4 and TSH concentrations returned to control levels within 24 hours while total T4

in serum subsequently declined in a time dependent manner. Normally, about 99.97% of T4 is bound to carrier proteins, and only 0.03% circulates as the unbound or free fraction. Free T4 is the bioavailable, or bioactive, fraction and it is in equilibrium with bound T4. For example, when serum total T4 is lowered, protein-bound thyroxine can act as a reservoir to maintain free T4 concentrations in the normal range. In this case,[84] it was suggested that PFOS competitively displaced thyroxine from its serum carrier proteins, thus temporarily increasing the free T4 concentrations and causing TSH to decline in response. The excess free T4 was then hypothesized to have been degraded at a higher rate, leading to low total T4, in order to maintain normal concentrations of free T4. This was supported by increases in $^{125}$I activity in urine and feces after a single oral dose of PFOS with $^{125}$I pre-treated rats.[84] This hypothesis was further explored and supported *in vitro* by Weiss *et al.*,[85] who examined competitive binding of perfluorinated acid compounds to the thyroid hormone transport protein, transthyretin. Here, binding potencies of twenty four different perfluorinated acids were tested in a radioligand binding assay. Competitive binding was evident, and binding potencies were highest for PFHxS>PFOS>PFOA. Researchers suggested that these effects may explain altered thyroid hormone concentrations as seen in previous animal studies.[85]

Other methods of increased T4 turnover by perfluorinated acids include evidence of increased excretion via glucuronidation from the Chang *et al.* study by increased liver UGT1A family mRNA transcription at 2 and 6 hours post-

administration of PFOS.[84] Given that total T3 was not as greatly reduced, T4 specific UGT isoforms such as UGT1A1 and/or UGT1A6 (in comparison to T3 specific isoforms such as UGT2A2) may have been selectively induced.[84] The lack of TSH increase could be explained by this hypothesis, in that rats with T4 specific UGT1A inducers did not have clinically significant TSH increases.[84] Instead, liver malic enzyme mRNA transcripts were also increased after 2 hours of a PFOS dose, indicating an increased liver tissue response to thyroid hormone.[84] Malic enzyme itself produces intermediates in the carbohydrate catabolism process thus hepatic malic enzyme activity is commonly used as an indicator of changes in thyroid hormone.[86, 87]

Although Butenhoff *et al.* did not report thyroid hormone levels in their study of PFHxS in rodents, male rats exhibited an increase in mild to moderate thyroid follicular and hepatocellular hypertrophy. [52] Interestingly, increased hepatocellular hypertrophy often occurs with increased thyroid follicular epithelial hypertrophy/hyperplasia. Thus, further evidence is presented that increased thyroid hormone turnover occurs through degradation in the liver (increased hepatocellular hypertrophy). [52]

Pituitary gland integrity as a result of perfluorinated acid exposure has also been investigated thoroughly. The lack of TSH response and the pituitary's involvement were examined by removing the pituitary glands of PFOS- treated rats and their cells subsequently cultured to monitor TSH secretions over a 12

hour incubation period.[84] Here it was shown that PFOS did not lower total T4 through the HPT axis and, therefore, did not interfere with TSH release.[84] This was demonstrated by the lack of effect from PFOS on TSH during dosing of TRH from static cultures in comparison to in vivo studies with reduced serum TSH secretions by propylthiouracil treatment (a goitrogen), alone or with PFOS.[84]

The HPT axis integrity, as a whole, was suggested as the mechanism of thyroid hormone disruption but this theory has been ruled out. One study showed that propylthiouracil caused a two- to three- fold increase in TSH in comparison to the small increases seen with the low PFOS dosage group (3 mg/kg), suggesting no significant compromise in the integrity of the HPT axis.[84] Here, TSH recovery despite serum T3 and T4 reductions indicated that the homeostatic balance of thyroid hormone may have been reset, similar to long term chemical disruption of the thyroid axis.[84] However, the higher PFOS dose group (5 mg/kg) had decreased TSH in the initial stages of exposure.[84]

It is notable that there has been considerable debate over the accuracy of thyroxine measurements in animals following perfluorinated acid exposure due to a possible analytical bias. Measurements of free T4 by "analog methods", such as a measurement of thyroid hormone binding ratios in serum, in samples containing PFOS are prone to a negative bias because PFOS competes with free T4 in the assay by binding to serum proteins.[88] An equilibrium dialysis reference method (ED-RIA) can be used to measure free T4 correctly.[88] In an experiment by Chang

*et al.* using ED-RIA, serum total T4 and TSH concentrations were not reduced after three daily 5 mg/kg oral doses of potassium PFOS [88]; despite that free T4 was apparently decreased using two common analog methods (chemiluminometric immunoassay and radioimmunoassay).[88] These data suggest that PFOS can displace free T4 and labeled free T4 analogs from serum binding proteins. Chang *et al.* suggest that a reference method, such as ED-RIA be used for free T4 determination when PFOS is present.[88] However, thyroid hormone analysis by ED-RIA is cumbersome, and testing for potential PFA interference with analog methods a priori may be considered adequate for accurate thyroid hormone concentrations. For example, Gutshall et al. tested concentrations of PFDA up to 100 µg/mL, and found that there was no interference with the radioimmunoassay for thyroid hormone analysis.[77] However, it is not clear if other PFAs cause the same negative bias as PFOS in analog T4 assays. Currently, PFAs have been shown to perturb thyroid metabolism genes, [28] displace T4 from its carrier proteins [43] and to affect total T4 concentrations despite unaffected free T4.[27] This current study makes a valuable contribution to the controversy in ruling out the possible hypothyroxinemic inducing effect of PFAs during pregnancy.

### 1. 5. 2. Human Studies

#### 1. 5. 2. 1. Implications of Maternal Hypothyroidism for Development

Maternal hypothyroidism is a condition defined by high maternal TSH concentrations. In developed countries, the most common cause for hypothyroidism in women of child-bearing age is chronic autoimmune

thyroiditis.[89] Here, antibodies which are responsible for maternal thyroid function, can cross the placenta and compromise fetal and neonatal thyroid function. [90-92]

Although not entirely conclusive, maternal hypothyroidism has been shown, although not clearly, to be related to reduced childhood intelligence quotient (IQ).[13, 89, 93]This proposed relationship has been noted for approximately 100 years. Man and Jones (1969) suggested that mild maternal hypothyroidism could be associated to lower IQ levels in children [94] while Matusuura and Konishi (1990) showed that fetal brain development was adversely affected by hypothyroidism in the mother and the fetus as a result of chronic autoimmune hypothyroidism.[95] Haddow *et al.* were able to show that hypothyroidism in pregnant women was related to attenuated neuropsychological tests in their children.[89]

### 1. 5. 2. 2. Implications of Maternal Hypothyroxinemia on Development

Unlike hypothyroidism, hypothyroxinemia has long been assumed to have no adverse consequences for both the mother and fetus. However, in 1999 Pop *et al.* reported the first study describing adverse relationships between maternal hypothyroxinemia and neuorodevelopment in children.[96] Here, Pop *et al.* screened maternal plasma free T4 concentrations during pregnancy and infant neurodevelopment in iodine sufficient areas.[97] Maternal free T4 concentrations below or equal to the 10$^{th}$ percentile (TSH within the reference range) at 12 weeks'

gestation were associated with developmental delays in infants at 10 months of age, as measured by the Bayley Scales of Infant Development.[97]

A later report by the same group examined child development in a prospective 3 year follow up study. Here, it was shown that mothers who had hypothyroxinemia in the first trimester, but whose free T4 levels increased during gestation (at 24 and 32 weeks), had children without any significant neurodevelopment delay at 24 and 48 months of age.[96] Higher concentrations of free T4 (between the 50$^{th}$ and 90$^{th}$ percentiles) in pregnant women during their first trimester did not cause any adverse normal neurodevelopment, even when the free T4 concentrations declined at 24 and 32 weeks of gestation.[96] It was concluded that an adequate supply of maternal free T4, early during pregnancy, was critical for the child's neurodevelopment.[96]

A more recent study, in 2006 demonstrated that hypothyroxinemic women, assessed at 12 weeks' gestation, had children who showed significantly lower scores on the Neonatal Behavioral Assessment Scale (NBA) orientation index in comparison to controls.[98] This study examined 108 neonates with mothers who were hypothyroxinemic (free T4 concentrations <10$^{th}$ percentile) in comparison to 96 neonates whose mothers had free T4 values between the 50$^{th}$ and 90$^{th}$ percentiles. It was further shown that first-trimester maternal free T4, and not maternal TSH or free T4 later on in gestation, was a significant predictor of the children's scores. This study brings greater evidence that early maternal

hypothyroxinemia is a risk factor for neurodevelopment, as its effects were demonstrated in infants as young as 3 weeks old. Thus previous and existing practices of exclusively examining TSH as an indicator of thyroid hormone disruption may be questioned, and looking at both TSH and free T4 concentrations may prove beneficial in identifying relevant risks early during pregnancy.

Kooistra *et al.* further strengthened the link between hypothyroxinemia and neurodevelopment by considering possible effects from confounding factors in their study. Pop *et al.*'s study examined children at 12 and 24 months of age, which in some regard may be confounded by psychosocial factors that may interfere with infant development. Kooistra *et al.* were able to show that neurodevelopmental delay could be directly related to maternal hypothyroxinemia in children at 3 weeks of age. A disadvantage in this study was that early neonatal behavior is unstable and difficult to assess consistently, although these tests have been shown to have great predictive value of neurobehavioral outcome within the first year of life.[98]

There has only been 1 long term prospective follow up study from children born to hypothyroxinemic mothers. These hypothyroxinemic women were from a moderately iodine deficient area and were defined with the condition by normal TSH concentrations and "low" serum free T4 concentrations compared to range values from women living in a moderately iodine sufficient area. [99] Children who

were born to healthy mothers from an iodine-sufficient area were compared to these children [99] Here, approximately 68.7% (11 of 16) of children from the iodine-deficient area developed attention-deficit/hyperactivity disorder (ADHD) by the age of 10, compared to no ADHD diagnoses for those born in iodine sufficient areas. The presence of ADHD in the iodine-deficient area was significantly higher ($p<0.01$) in children born to mothers who were hypothyroxinemic.[99] Hauser *et al.* had previously demonstrated that 70% of children who had resistance to thyroid hormone (impairment of thyroid function) were found to have ADHD.[100] Indeed, low scores on the orientation cluster of Neonatal Behavioral Assessment Scales (NBAs) have the propensity to be interpreted as an early marker for ADHD.[98]

### 1. 5. 2. 3. Epidemiology of Perfluorinated Acids

A number of biomonitoring studies across the world have been conducted on perfluorinated acids but relatively little research has been done on the respective epidemiology of this exposure. At the time this thesis research was undertaken, only occupational perfluorinated acid exposure studies had been reported, there were no published studies on the effects of perfluorinated acids in background populations. However, new studies on the epidemiology of background populations have recently been published. These occupational and non-occupational studies will be briefly reviewed.

### 1. 5. 2. 3. 1. Occupational Studies

Biennial medical surveillance of fluorochemical production workers at the 3M Company (Decatur, Alabama and Antwerp, Belgium) were examined by Olsen *et al.* in 1995 (n=178 male employees) and 1997 (n= 149 male employees).[101] Mean serum PFOS concentrations were as follows: 1995, 2.19 parts-per-million (ppm) and 1997, 1.75 ppm. Here, no significant changes in serum hepatic enzymes, cholesterol, or lipoproteins were observed for employees with PFOS concentrations less than 6 ppm (95% of serum PFOS concentrations). In this study, concentrations were stratified into four categories and those over 6 ppm were examined in order to determine if any effects were evident at higher PFOS concentrations. However, effects were not conclusive for those employees with PFOS concentrations greater than 6 ppm. This was due to the lack of Antwerp subjects having concentrations greater than 6 ppm of PFOS in 1997.

Another report examining PFOS effects included a retrospective cohort mortality study on employees of a perfluorooctanesulfonyl fluoride manufacturing plant (PFOS and its related precursors are all derived from perfluorooctanesulfonyl fluoride).[102] Here, workers with at least one year of cumulative employment at the facility were followed (n=2083, 83% males). Although 65 deaths were reported amongst the cohort, these mortality rates were lower than expected based on rates in the general population. Additionally, three deaths from bladder cancer were reported amongst workers who had high exposures, but it was uncertain if these occurrences were related to fluorochemical exposure, as these cases were from

workers in non-production jobs such as maintenance, incinerator, and wastewater treatment plant operations.[102]

Another study by Olsen *et al.* examined male ammonium perfluorooctanoate (salt of PFOA) production workers during 1993 (n=111), 1995 (n=80), and 1997 (n=74).[103] In this study, mean serum concentrations of PFOA were: 1993, mean 6.6 ppm, SD 12.2, 1995, mean 6.8 ppm, SD 16.0, 1997: mean 6.4 ppm, SD 14.3. Plasma cholecystokinin, hepatic enzymes, cholesterol and lipoprotein concentrations were measured and it was concluded that there were no significant clinical hepatic toxicities associated with PFOA concentrations.

A more recent study examined employees of a perfluorononanoic acid (PFNA) surfactant blend plant (n=630) between January 1 1989 and July 1 2003.[104] Work histories were available for exposure determination. Five cross-sections at a time (1976, 1989, 1996, 1998 and 2001) were taken, as were 32 clinical chemistry variables including total cholesterol, alkaline phosphatase, bilirubin, triglycerides, calcium, phosphorus, glucose, iron, etc. Concentrations were divided into three categories: high, low, or no exposure. Exact numerical values of the cutoffs for the categories were not given but it was shown that there were no significant changes by unit increase in cumulative exposure intensity scores. Thus PFNA through a PFNA surfactant blend had no adverse clinical effects through occupational exposure.

### 1. 5. 2. 3. 2. Non-occupational Studies

Most of the non-occupational perfluorinated acid epidemiology research has focused on outcomes such as low birth weight.. Comparatively little has been done on human thyroid hormone outcomes, such as hypothyroidism, and no reports have specifically examined hypothyroxinemia; some reports have focused on perfluorinated acid effects on thyroid hormone concentrations, but not hypothyroxinemia specifically.

### 1. 5. 2. 3. 3. Birth weight

Low birth weight from perfluorinated acid exposure is an outcome that has recently garnered attention. Studies on rodents with PFOA have demonstrated developmental toxicities such as reduced fetal weight and postnatal survival, as well as delays in postnatal growth and offspring development.[34, 105, 106] PFOS exposure in rats and mice has been involved with developmental and reproductive toxicities such as birth weight reduction, gestational length decrease, structural defects, developmental delays and increased neonatal mortality.[54, 55, 57, 62, 66, 107]

Despite such repeated outcomes from animal studies, results from the human population have been varied and few. One occupational study reported no association between job employment with high PFOS exposure before the end of pregnancy, and birth weight.[108] Non occupational studies have been more abundant than occupational ones. Apelberg *et al.* (2007) found small negative

associations with increasing PFOS and PFOA and birth weight, after adjusting for confounding factors (n=293).[23] In contrast, Fei *et al.* reported that maternal PFOS concentrations were not associated with fetal growth indicators, suggesting that PFOA and not PFOS, may affect organ and skeletal growth (n=1400).[109] Further studies in a Danish national birth cohort also showed inverse associations with PFOA concentrations and birth weight (n=1400).[110] However, Washino *et al.* reported that PFOS, not PFOA, levels were negatively associated with low birth weight in a hospital based prospective study (n= 428 women).[111] The first study in Canada showed no association between serum PFCs during 24-28 weeks, at delivery (n=101), and in cord blood serum (n=105) and birth weight.[112] Researchers concluded that although concentrations were ubiquitous, perfluorinated acid exposure did not demonstrate effects on birth weight.[112] Another recent Canadian study by Hamm et al. (in press) similarly, did not show any relationship between perfluorinated acid exposure and low birth weight. Here, maternal exposure to three different perfluorinated acids did not have an influence on the risk for delivering a small for gestational age (SGA) infant: PFOA: 1.15 (0.49-2.69), PFHxS: 1.54 (0.91-2.63), and PFOS: 0.80 (0.50-1.28) (per log unit).

A recent commentary furthermore questioned the usage of birth weight as an indicator of human toxicity, because the subtle decreases in birth weight may fall in between the high standard deviations of birth weight variability.[113] These shifts in birth weight may reflect variation within the normal range of the distribution, even if relations are significant.[113] For example, Savitz interpreted the significant

changes in birth weight with PFA exposure in the Apelberg et al.[23] and Fei et al.[110] studies as being shifts within the overall distribution of birth weight distribution, but having no "enhanced effect on the tail." Thus because of the variation in PFA exposure and modest changes in birth weight in these two studies, the possible relationship between PFA exposure and birth weight is still not clear. Nonetheless, we argue that given limited studies in this area and possible measurable effects on human physiology, the underlying biochemical mechanisms, possibly involving hormones, are worthy of study.

## 1. 6. Perfluorinated Acids and Thyroid Effects

One study conducted by Emmett *et al.*indirectly examined the relationship between perfluorinated acid concentrations and thyroid disease.[114] More specifically, certain biomarkers of toxicity and/or a past liver/thyroid disease were associated with PFOA concentrations in a community of people with long term chemical exposure due to a fluoropolymer production facility neighbouring the community. The 371 residents were selected through stratified random sampling and a lottery among the volunteers. In addition to cholesterol, red cell indices, white cells and platelet counts, and thyroid stimulating hormone were also examined (but not thyroid hormone concentrations). None of the examined parameters were associated with PFOA and it was concluded that there was no apparent toxicity from PFOA using these parameters. It is important to note that because thyroid hormone concentrations were not examined that this lack of an

association between PFOA and TSH may not be surprising given what we know from animal studies. A study by Inoue *et al.* examined PFOA and PFOS concentrations as well as thyroid stimulating hormone and free T4 in pregnant women (n=15, age 17-37) living in Japan.[24] This study did not show any association between free T4 or TSH and PFOS levels in maternal and cord blood in pregnant women (n=15). While this may be indicative of a lack of a relationship between PFOS and hypothyroxinemia, the authors admitted that the sample size may have been too small to show a significant effect. [24]

A recent report by Peck *et al.* studied various persistent organic pollutants (10 polybrominated diphenyl ethers, 35 polychlorinated biphenyls, 9 persistent pesticides, 7 perfluorinated acids) and thyroid hormone concentrations (total T4, total T3, free T4 and thyroid stimulating hormone) during pregnancy (n= 42 who were at less than or equal to 22 weeks gestation) in Central Oklahoma.[115] Perfluorinated acids were not shown to be associated with reduced free T4 concentrations. However, women with higher PFOS concentrations were associated with TSH increases of 0.7 mU/L. Thus indications of hypothyroidism are suggested, but no biological plausibility exists from toxicology to explain this.

## 1.7. Overview and Justification for Thesis Work

As evidenced by the above review, the toxicology of PFOA and PFOS are rather well documented today, including in pregnant animal models. A relatively

consistent finding in these studies has been effects on birth weight and the disruption of maternal thyroid hormones, without a concomitant effect on TSH, raises questions about whether perfluorinated are contributing to hypothyroxinemia in background human populations. Although no studies on the epidemiology of perfluorinated acids for the background population were available at the onset of this thesis, a few reports are now available on the effects of perfluorinated acids on birth weight and some have also examined effects on thyroid endpoints. To date however, no study has specifically examined if perfluorinated acids are determinants of maternal hypothyroxinemia.

Although PFHxS is the third most abundant perfluorinated acid found in humans and the environment, toxicology and epidemiology studies have generally only reported on PFOA and PFOS. It is not clear why the epidemiology studies have not reported on PFHxS, but this may be due in part to intereferences that arise in the quantitative analysis of PFHxS in human serum. A previous report in our lab identified the specific endogenous hormones that can interfere in the analysis of PFHxS in human serum and offered a potential solution to avoid these, but this solution was not validated and furthermore the effects of either pregnancy or gender were not examined. Therefore, in Chapter 2 we examine the interferences from endogenous steroid sulphates that may cause substantial over-reporting, or non-reporting, of PFHxS in past research. Additionally, we provide evidence that this may be especially problematic for samples from pregnant women. Approaches to avoid this systematic analytical bias are validated.

In Chapter 3, the improved methods developed in Chapter 2 are applied to a case-control study design whereby we examined whether PFOS, PFOA or PFHxS concentrations are predictors of hypothyroxinemia in a pregnant population selected from Edmonton, Alberta, Canada (n=270).

Finally, the public health significance from these results and other related effects from perfluorinated acid exposure are discussed in Chapter 4.

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# CHAPTER 2. Endogenous HPLC-MS/MS Interferences and the Case of Perfluorohexane Sulfonate (PFHxS) in Human Serum; Are we overestimating exposure?

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**Abstract**

Perfluorinated acids have received increasing scientific attention due to their widespread global distribution, environmental persistence and bioaccumulation in wildlife and humans. For perfluorohexane sulfonate (PFHxS, $C_6F_{13}SO_3^-$, *m/z* 399), all existing human data have been generated using HPLC and its most sensitive MS/MS transitions (*m/z* 399/80 $[SO_3]^-$ or *m/z* 399/99 $[SO_3F]^-$), but this may be problematic because of co-eluting endogenous steroid sulphates that share common fragmentation pathways. We examined the magnitude of over-reporting for PFHxS in pregnant women (n=29), and in pooled serum of males, non-pregnant and pregnant females (n=3, 100 samples per pool) by comparing *m/z* 399/80 and 399/99 data to an interference free transition, *m/z* 399/119. PFHxS concentrations in pregnant women determined using *m/z* 399/80 and *m/z* 399/99 ($p<0.05$), but not *m/z* 399/119, were positively correlated to the response of the steroid sulphates. This led to an average overestimation of PFHxS by 1.5 and 4.7-fold, using *m/z* 399/80 and 399/99, respectively, and validated the use of *m/z* 399/119 for the first time. The interferences were problematic in all human serum samples, and analysis of pooled serum revealed statistically significant over-reporting by *m/z* 399/80 and 399/99 for pregnant women > non-pregnant women > men. The magnitude of over-reporting here represents a worst-case scenario, but the extent to which published literature are biased is unknown due to limited details of methods in existing reports. Instead of using the less sensitive *m/z* 399/119 transition, we showed that an alternative selection of column and mobile phase can allow for sufficient chromatographic separation of the interferences. In

conclusion, it was shown that routine analytical methods are prone to systematically overestimating PFHxS concentrations in serum of men or women, but that this can be avoided by alternative chromatographic steps or MS/MS transitions.

## 2.2. Introduction

Perfluorinated acids (PFAs) are emerging contaminants that are receiving increased scientific scrutiny due to their global distribution,[1] environmental persistence,[2, 3] and chain-length dependent bioaccumulation potential. [4] Perfluorooctanesulfonate (PFOS) is the major PFA and currently among the most prominent of all contaminants in blood of humans or wildlife.[1, 5, 6] In rodents, PFOS may cause liver hypertrophy and decreased serum cholesterol and triglycerides.[7, 8] *In utero* exposure results in developmental defects [9] and a dose-dependent decrease in postnatal survival. [10] There is comparably little toxicological data available for perfluorohexane sulfonate (PFHxS) despite that PFHxS is also a major PFA in human blood [11] and has a longer pharmacokinetic half-life than PFOS (8.5 years, compared to 5.4 years for PFOS).[12] PFAs have had many historical uses in commercial products or as industrial chemicals, and still find use in the manufacturing of fluoropolymers. They are also degradation products of perfluoroalkyl surfactants and stain repellents used for coating paper, carpet, and textiles. In general, however, the sources of human PFA exposure and the associated health risks are poorly defined. To enable accurate exposure monitoring and risk assessments, highly specific and accurate analytical techniques are required for PFAs.

Quantitative analysis of PFAs in serum has most often been conducted using ion-pair extraction (IPE), or solid phase extraction (SPE), followed by high

performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) [10, 11, 13-27]. The challenges of PFA analytical methods have been reviewed, [28] but for PFOS in particular, the very first report of it in human serum[11] noted the presence of a coeluting MS/MS interference in the most sensitive MS/MS transition (i.e. *m/z* 499/80) – later identified as taurodeoxycholate isomers [29] – which could cause significant over-reporting of concentrations. For PFHxS ($C_6F_{13}SO_3^-$; *m/z* 399), almost all existing data has been generated using its most sensitive MS/MS transitions: *m/z* 399/80 ($[SO_3]^-$) or *m/z* 399/99 ($[SO_3F]^-$).[11, 19-21, 23-25, 27, 30] However, it is now unclear whether the existing PFHxS data may be systematically biased as a result of coeluting endogenous interferences in pooled maternal serum which share both of these MS/MS transitions [29]: the major source being 5-pregnan-3,20-diol-2-sulphate, commonly known as pregnandiol sulphate, and the minor source being $^{34}$S-3-hydroxy-5pregnan-20-one sulphate, commonly known as isopregnanalone sulphate.

We initially reported that these caused a 10 to 20-fold over reporting of PFHxS in pooled maternal serum,[29] but it remains unclear how widespread these interferences are in individual maternal serum samples, in men, or in non-pregnant women. Other investigators have also noticed evidence for these PFHxS interferences in certain human samples. For example, Apelberg et al.chose not to report their PFHxS data due to apparent interferences in human cord serum,[31] whereas others have combined the area counts of unresolved peaks in these PFHxS transitions; [24] possibly assuming that these were branched PFHxS isomers.

Furthermore, in an interlaboratory study, PFHxS concentrations had the greatest variability (up to 33.1% coefficient of variation) among all polyfluorinated analytes reported.[25] Consequently, there is some evidence that published human PFHxS serum concentrations [11, 13-26, 31, 32] may be over-reported, but the extent to which this is a problem is unknown. Here we examined the magnitude of PFHxS over-reporting in human serum by SPE- or IPE-HPLC-MS/MS in individual and pooled serum samples.

## 2. 3. Experimental

### 2. 3. 1. Human Serum Samples

Pooled male serum (n=3, 100 individuals per pool) and pooled non-pregnant female serum (n=3, 100 individuals per pool) were obtained from Golden West Biologicals, Temecula, CA, USA. Additional pooled maternal serum (n=3, 150-200 individuals per pool) and individual samples were collected from pregnant women during the $15^{th}$-$16^{th}$ weeks of pregnancy in 2005 in Edmonton, AB, Canada. Ethics approval was obtained from the University of Alberta Health Research Ethics Board for collection of all samples in Edmonton. All samples were frozen at –80° C until analysis.

### 2. 3. 2. Chemicals and Reagents

Formic acid (50 %, HPLC grade) and PFHxS (>98% purity) were acquired from Fluka (Oakville, ON, Canada). Methanol (HPLC grade), methyl-*tert*-butyl ether (MTBE; HPLC grade), ammonium hydroxide (Optima grade) and sodium hydroxide (>97% purity) were obtained from Fisher Scientific (Ottawa, ON, Canada). Tetrabutylammonium hydrogen sulphate (>97% purity) and sodium carbonate (>99.5% purity) were purchased from Sigma-Aldrich (Oakville, ON, Canada). $^{18}$O-PFHxS was obtained from Wellington Labs (Guelph, ON, Canada). Reagent grade water was prepared by a Millipore Milli-Q system (Bedford, MA,

USA), and all reagents were stored at room temperature and made fresh every 2 weeks.

### 2. 3. 3. SPE of Serum

SPE extraction of serum was performed by a methodology similar to Kuklenyik et al.[24]. Briefly, samples were prepared by adding 5 ng of internal standard ($^{18}$O-PFHxS) and 3 mL of 0.1 M formic acid to 0.5 mL of serum in a 15 mL polypropylene tube and sonicating for 20 min. Oasis-HLB columns (200 mg, 6cc) (Waters Corp, Taunton, Massachusetts, USA) were pre-conditioned with HPLC grade methanol (6 mL) and 0.1 M formic acid (6 mL). The serum sample was loaded quantitatively onto the cartridge using 1 mL of 0.1 M formic acid. The cartridge was then washed with 6 mL of 50% 0.1 M formic acid in methanol, followed by 1 mL of 1% ammonium hydroxide in water, and subsequently vented with air for 5 min. Analytes were eluted with 6mL of HPLC grade methanol into clean 15 mL polypropylene tubes, and the cartridge then vented with air for another 10 min to collect all eluent. The eluent was concentrated under a stream of dry nitrogen to 0.5 mL and brought up to volume, if necessary, with methanol. The samples were centrifuged for 10 min at 4000 rpm to remove suspended particles, and 250 μL of supernatant was gently transferred to 0.75 mL polypropylene HPLC vials, vortexed for 1 min, and stored at 4°C until analysis.

## 2. 3. 4. IPE of Serum

IPE extraction of serum was performed by a methodology similar to Hansen et al..[11] Samples were prepared by combining 0.5 mL of serum, 1 mL of tetrabutylammonium hydrogen sulfate (0.5 M, adjusted to pH 10 with concentrated sodium hydroxide), and 2 mL of sodium carbonate in 15 mL polypropylene tubes. A 5 mL aliquot of MTBE was added and samples were shaken for 12 min, centrifuged, and 4.5 mL of the MTBE was transferred into a second polypropylene tube. This was repeated twice more, and the combined MTBE eluent was evaporated to 200 μL using a stream of dry nitrogen. The final MTBE concentrates were evaporated to dryness at room temperature and reconstituted in 500 μL of methanol of which 450 μL was finally transferred to a 0.75 mL HPLC vial and stored at 4°C until analysis.

## 2. 3. 5. Analysis by HPLC-MS/MS

PFA and steroid sulphate interference data were collected with an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA, USA) coupled to a hybrid triple-quadrupole linear ion trap mass spectrometer (4000 QTRAP, MDS Sciex, Concord, ON, Canada) equipped with an electrospray interface operating in negative ion mode. Chromatographic separation was accomplished by injecting 10 μL of sample extract onto a guard column connected to a Zorbax C8 column (3.5 μM, 2.1 mm x 5 cm, Agilent Technologies, Palo Alto, CA, USA). A reverse phase Zorbax C18 column (5μm, 4.6mm x 15cm, Agilent Technologies, Palo Alto,

CA, USA) was placed upstream of the injector to trap and separate instrumental PFA contamination. Gradient elution consisted of 10 mM ammonium acetate (pH = 6.4) as solvent A, and 100% methanol as solvent B at a constant flow rate of 0.18 mL/min. Starting conditions were 80% A, 20% B. The gradient was ramped linearly to 30% A and 70% B by 1 minute, and 100% B by 8 minutes, followed by an 11.5 minute hold and returning to starting conditions by 21 minutes. Initial conditions were held for a further 19 minutes to allow for column equilibration, resulting in a total run time of 40 minutes. Infusion of PFHxS native standard was used to optimize instrumental parameters including nitrogen curtain gas (20 L/min), nitrogen collision gas (12 L/min), ion spray voltage (4500 V), entrance potential (-10 V), collision exit potential (-15 V), source temperature (350°C) and ion source gas (50°C). Multiple reaction monitoring (MRM) was used to monitor multiple MS/MS transitions. Instrument parameters for optimal analysis of the interferences were tuned using enhanced product ion scans over a range of declustering potentials and collision energy; the parameters which resulted in the maximum signal to noise ratio were adopted for further analysis.

As a comparison to the above analytical method, a select number of samples were also analyzed on a Synergi hydro-RP 80A column (4μm, 3.00 mm x 150 mm, Phenomenex, Torrance, CA, USA) in an independent laboratory (ALS Laboratory Group, Edmonton, AB). Gradient elution consisted of 20 mM ammonium acetate (pH 4) as solvent A and 100% methanol as solvent B. Initial conditions were 60% A and 40% B, where solvent B was ramped to 80% at 3 minutes and held for 5

minutes. Solvent B was then increased to 100% at 8.5 minutes and held until returning to initial conditions at 11.5 minutes. Starting conditions were kept for another 6 minutes, allowing for a total run time of 17.5 minutes. Ionspray voltage was optimized at -3500V and column temperature was kept at 40°C.

## 2. 4. Results and Discussion

### 2. 4. 1. PFHxS Concentrations in Serum of Pregnant Women

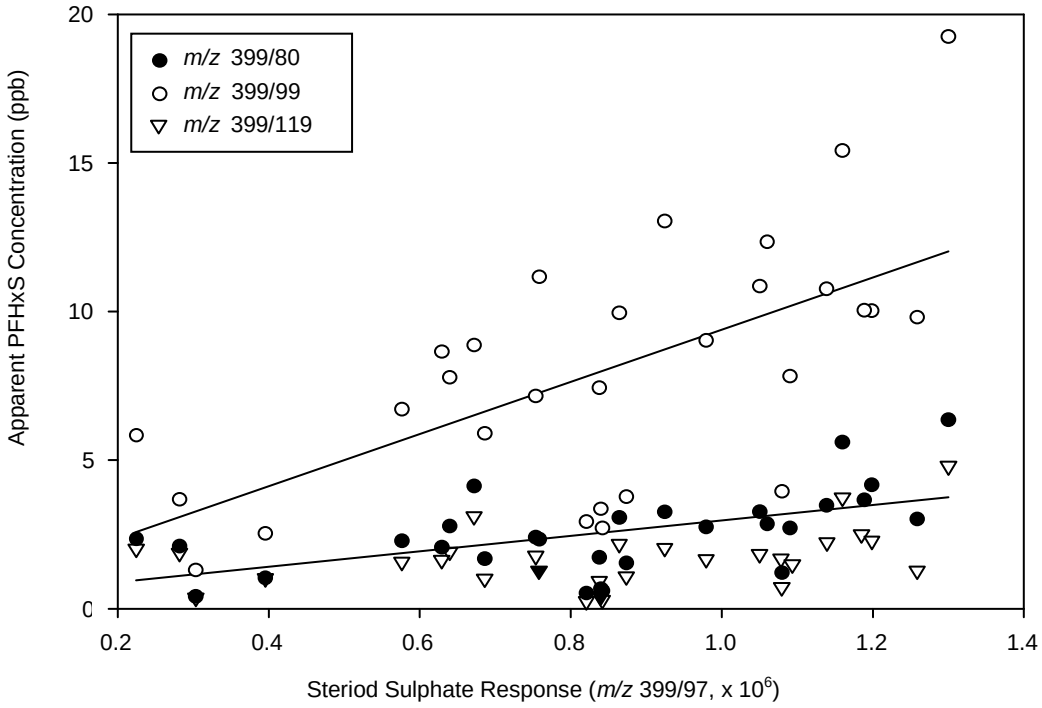


**Figure 2-1 Apparent concentrations of PFHxS in individual pregnant female serum samples (n=29). PFHxS was quantified by *m/z* 399/80, 399/99, and 399/119 versus total response of steroid sulphates, quantified by *m/z* 399/97. The *m/z* 80 and *m/z* 99 product ions were positively correlated ($p<0.05$; regression lines shown) with response of the steroid sulphate, whereas no association was observed for the *m/z* 119 product ion ($p>0.05$).**

We previously reported that, in pooled serum of pregnant women, the PFHxS concentrations determined in the *m/z* 399/99 and 399/80 transitions resulted in higher concentrations than when the *m/z* 399/119 transition was used.[29] To determine if this was also true for most individual serum samples of pregnant women, we separately analyzed 29 maternal samples. The apparent mean concentrations obtained using *m/z* 399/99 (8.0 ± 4.1 ppb,) and *m/z* 399/80 (2.6± 1.4 ppb) were both significantly greater ($p<0.001$) than for concentrations obtained using *m/z* 399/119 (1.7± 1 ppb). Apparent concentrations in *m/z* 399/99 were also significantly greater than for 399/80 ($p<0.001$). Consequently, the extent of over-reporting was 4.7-fold when using *m/z* 399/99 and 1.5 fold when using *m/z* 399/80, compared to *m/z* 399/119.

We previously hypothesized that the reason for the apparently higher concentrations of PFHxS, when using the *m/z* 80 or 99 product ions, was the presence of co-eluting endogenous steroid sulfates sharing common product ions. To study this hypothesis, we examined for correlations between sample response of the interference (collected in the *m/z* 399/97 transition, specific to the interference) and PFHxS concentrations in the same 29 individual serum samples from pregnant women (Figure 1). PFHxS concentrations determined using the *m/z* 399/80 and *m/z* 399/99 transitions were positively correlated to the response of the steroid sulphates ($p<0.05$). Conversely, there was no statistically significant association with the interference when PFHxS was quantified in the *m/z* 399/119 transition. Overall, these findings indicated that the steroid sulphates indeed

contributed to over reporting of PFHxS when the *m/z* 80 or 99 product ions were used for quantification, and as previously suggested, that the *m/z* 399/119 was an interference-free transition that could be used for accurate data collection.

## 2. 4. 2. Relative Extent of Interferences in Pooled Male, Pregnant, and Non-Pregnant Female Serum

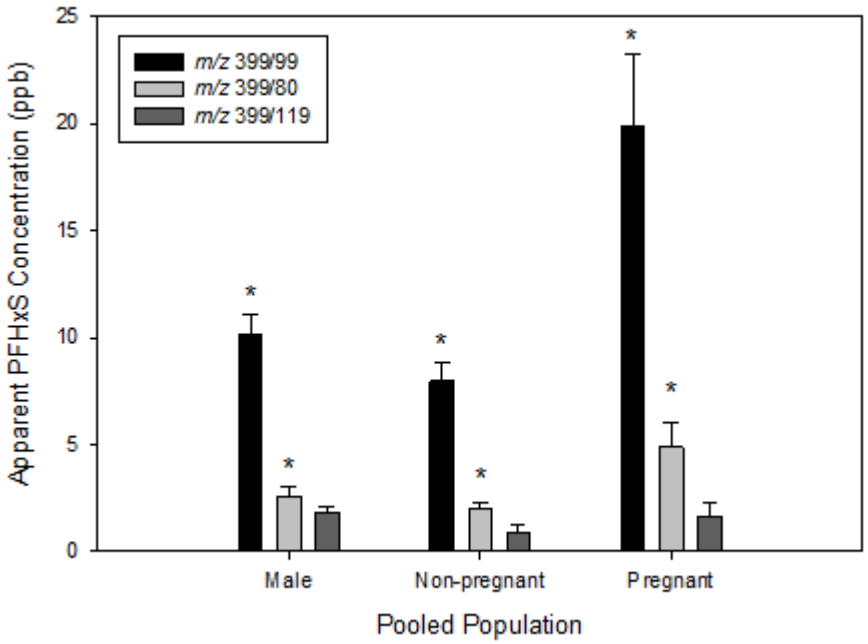


**Figure 2-2 Apparent concentrations (+1 standard deviation) of PFHxS in n=3 pooled serum samples. PFHxS was quantified with *m/z* 399/99, *m/z* 399/80 and *m/z* 399/119 for male, non-pregnant and pregnant females. * denotes a significant difference ($p<0.05$) relative to *m/z* 399/119 for each group**

Concentrations obtained by *m/z* 399/99 and *m/z* 399/80 were significantly higher than for *m/z* 399/119 in all pooled serum samples (males, pregnant and non-

pregnant female). Serum of pregnant women had the highest apparent PFHxS concentrations ($p<0.05$) using either *m/z* 399/99 or 399/80 compared to men or non-pregnant women. Males and non-pregnant females showed similar concentrations when using *m/z* 399/99 and *m/z* 399/80 ($p>0.05$), but when using the accurate *m/z* 399/119 transition it was evident that non-pregnant women had significantly lower concentrations than males ($p=0.028$). These trends can be explained by the total response of steroid sulphate interferences in these pooled samples. Pregnant females had significantly higher interference response than either males or non-pregnant females ($p<0.05$); 26 times more than males, and 6.9 times more than non-pregnant females. Non-pregnant females also had significantly greater response of the interferences than males ($p<0.05$). Taken together, these results demonstrate that: i) the steroid sulphate interferences are common to males and females, thus it is probable that most existing PFHxS data systematically overestimate the true extent of human exposure and, ii) that the contribution of the interference to the signal is enough to mask the true variation of exposure in human sub-populations.

The fact that serum of pregnant women contained the most steroid sulphate interference is not surprising. These steroid sulphates include pregnandiol sulphate and isopregnanalone sulphate.[29] Isopregnanalone is a precursor of progesterone, while pregnandiol is a degradation product of progesterone, a critical hormone that increases during the luteal phase of the menstrual cycle, and which increases even more during pregnancy. Isopregnalone and pregnandiol can

undergo sulphation in the liver to enable their excretion as polar conjugates.[33, 34] In humans, concentrations of isopregnanolone and its conjugates increase significantly during pregnancy[35] while increasing sulphation of this compound occurs after the 30$^{th}$ week of pregnancy.[36] Pregnenolone sulphate, the major precursor to epitestosterone, is commonly detected in males with levels peaking at approximately 27 years of age and then later remaining constant.[37] Concentrations of these sulphated steroid conjugates are on average 40-100 fold higher than the parent steroids.[36] Similarly, concentrations of isopregnanalone increase about three-fold in females compared to males during the luteal phase of the menstrual cycle[35] and concentrations of steroid sulphates have been shown to vary by sex or by external stimuli, such as stress.[36] Although we do not have information on the exact phase of these non pregnant women's menstrual cycles in our study, the phase dependent increase may have been diluted as these were pooled sera, thereby making the non-pregnant women and male PFHxS concentrations seemingly similar in the *m/z* 399/80 or 399/99 transitions.

Although we did not examine PFHxS interferences in human cord blood, Apelberg et al.reported interferent compounds that eluted at the same time as PFHxS which prevented PFHxS quantification.[31] Umbilical cord venous progesterone increases with longer duration of labour[38] and is dependent on the mode of delivery.[39] In fact, the placenta produces 250 mg of progesterone per day as the pregnancy progresses[40] and 75 mg per day is passed to the fetus through the umbilical vein.[41] Thus, natural or induced increases in progesterone are likely to

lead to the same difficulties in measuring PFHxS in cord serum, given progesterone's relationship with pregnandiol and isopregnanolone.

### 2. 4. 3. Practical Solutions

Clearly, the most immediate and simple solution to avoiding the steroid sulphate interferences when analyzing for PFHxS is to utilize the *m/z* 399/119 transition which was validated here to be unaffected by these steroids. Although this transition is not as sensitive as either of the major *m/z* 399/80 or 399/99 transitions, most modern instrumentation should be capable of meeting the detection limits required for the analysis of PFHxS in human serum. Using our instrumental conditions, the detection limits for PFHxS were 0.026, 0.072, and 0.16 ng/mL for *m/z* 399/80, 399/99, and 399/119, respectively (based on the concentration of standard injected that produces a signal to noise ratio of 3). If a more sensitive analysis is required, then the interferences must be separated chromatographically. Unfortunately, most routine PFA methods are not capable of effectively resolving PFHxS from its interferences.

In the current work, the interference peaks appearing in the *m/z* 99 and 80 product ion chromatograms were sometimes partially resolved from the linear PFHxS peak, as shown in Figure 3a. However, this was not consistent, and even at the best of times was insufficient for accurate integration. Furthermore, it was always ambiguous whether some of the additional peaks were truly interferences, or

possibly branched isomers of PFHxS; thus the entire hump was normally integrated. This is a realistic strategy, as described by Kuklenyik et al.who observed a shoulder peak adjacent to the PFHxS peak and combined the two unresolved peaks for quantification.[24] The likelihood that these steroid sulphate interferences may be misidentified as PFHxS branched isomers could depend on whether the analyst is using an electrochemical PFHxS standard (eg. Fluka, Oakville, ON, Canada) or a linear standard (e.g. Wellington Labs, Guelph, ON, Canada).

We previously presented a highly selective chromatographic method, employing a perfluorooctyl stationary phase, that separated the interferences from PFHxS by over 10 minutes[29]. However, these specialized perfluorinated columns are more expensive than most reversed phase columns, and the time required for a single analysis of all PFAs can exceed 1 hour. When we asked an independent laboratory to confirm the presence of the *m/z* 80 and 99 interferences, we serendipitously discovered an alternative chromatographic method that could separate the interferences in a relatively fast time-frame (Figure 3b). This alternative method utilized a Synergi hydro-RP 80A column, and a gradient elution program comprising 20 mM ammonium acetate (pH 4) and 100% methanol as mobile phases. A separate set of pooled serum samples from women in Alberta (n=99, 150-200 individuals per pool) were analyzed by this new method.

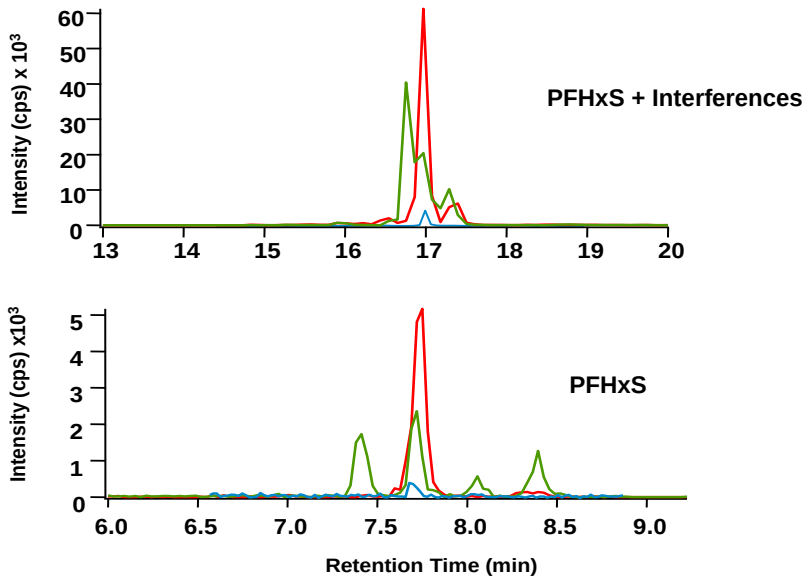


**Figure 2-3 Extent of chromatographic separation of the interferences.**
**(A) C8 and (B) Synergi hydro-RP, showing *m/z* 399/99 (green), *m/z* 399/80 (red) and *m/z* 399/119 (blue). PFHxS in Figure B is the peak at 7.7 minutes, while other peaks are the interference.**

Chromatograms produced by this alternative method were similar in that most interference was observed in *m/z* 399/99, and this method confirmed that the *m/z* 399/119 transition appeared to be interference free (Figure 3b). With this new method it was more reasonable to attempt to integrate the PFHxS peak in *m/z* 399/99 or *m/z* 399/80, owing to better separation from the interference peaks which eluted before and after PFHxS. Mean concentrations obtained from 399/80 and 399/99 were not significantly different by this method ($p>0.05$), thus demonstrating the effectiveness of interference separation. Similar to results on the C8 column, if the total peak area was artificially integrated (i.e. for all peaks eluting in the vicinity of PFHxS), resulting concentrations were 3.5-fold larger

when peaks were combined for *m/z* 399/99 ($p<0.001$) and 1.1-fold ($p=0.02$) larger when *m/z* 399/80 peaks were combined together, compared to *m/z* 399/119.

Interlaboratory studies have previously reported discrepancies in PFHxS concentrations. In Longnecker *et al.*[25], six laboratories used HPLC-MS/MS with an assortment of sample preparation methods including weak anion exchange (WAX), protein precipitation, liquid-liquid, and ion pairing extraction. Coefficients of variation among these six laboratories were reported at 33.1% for PFHxS.[25] Interestingly, all six laboratories utilized *m/z* 399/80 while two others also included *m/z* 399/99 or *m/z* 399/119 in their methods, although comparison of results from the different transitions was not reported. In a larger interlaboratory study,[42] coefficients of variation among 18 participating laboratories were as high as 64% for PFHxS while other PFAs such as PFOS were lower (32%). Variation was attributed to the various matrices that were used, such as fish tissue, fish liver, and water, but not to any other specific cause. It is possible that the varying sample preparation methods could also have contributed to the variable PFHxS concentrations if some methods were better at co-extracting steroid sulphates from the biological samples. To examine for this possibility we compared pooled samples of male, and pregnant or non-pregnant females, extracted by IPE or SPE ($n=3$ for each pooled sample type and extraction type), but the steroid sulfate response was not statistically different between SPE or IPE extraction for any pooled samples. Thus, it is unlikely that the sample preparation technique would influence the extent of the systematic bias.

## 2. 5. Conclusions

PFA research has generally found that PFHxS is the third most abundant PFA in humans. We have hereby demonstrated that the most common quantification techniques for PFHxS, which rely on HPLC and *m/z* 399/99 and 399/80 MS/MS transitions, are prone to over reporting in serum samples of women and men due to co-eluting steroid sulphate interferences. The extent of the bias was shown to be sex and pregnancy dependent but was present in all samples examined, and furthermore obscured the true variation in human sub-population exposure to PFHxS. The magnitude of over reporting here should be viewed as a worst case scenario. The extent that published data may be affected depends on various factors such as the use of matrix matched standards, the identity and number of transitions monitored, and the degree of chromatographic separation. Because these factors are seldom discussed in methodologies, the analytical bias in previous reports is unknown. Two simple solutions were validated to avoid over reporting of PFHxS concentrations in future studies: 1) using the interference free, yet less sensitive *m/z* 399/119 transition and 2) using an alternate chromatographic method to achieve greater resolution of the interferences from PFHxS. Although only human serum was examined in the current work, other matrixes may also contain the steroid sulphate interferences, such as waste water treatment plant influent and effluent, thus wider caution should be exercised for all samples in future studies.

**Acknowledgements**

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# CHAPTER 3. Perfluorinated Acid Concentrations as Determinants of Maternal Hypothyroxinemia.

## 3. 1. Introduction

Perfluorinated acids (PFAs) and their precursors have been manufactured for over 50 years for applications in hundreds of consumer and industrial applications including stain resistant coatings for textiles and paper, and in fire fighting foams. [1] In 2001, PFAs began to draw increasing attention when they were discovered to be widespread in humans [2] and wildlife [3] owing to their long production history, environmental persistence, and chain-length dependent bioaccumulation.[4]

Most of the early research concerning PFAs and human health were occupational studies conducted by the manufacturing industry, with few health effects reported. For example, Olsen et al.examined fluorochemical production workers at the 3M Company (Decatur, Alabama and Antwerp, Belgium) in 1995 (n=178 male employees) and in 1997 (n= 149 male employees).[5] Mean perfluorooctane sulfonate (PFOS) concentrations were: 1995, 2.19 parts-per-million (ppm), and 1997, 1.75 ppm. Subjects were stratified into four categories of PFOS exposure concentrations (0 to <1 ppm, 1 to 3ppm, 3 to <6ppm, ≥ 6ppm) and no significant changes in serum hepatic enzymes (ie. gamma-glutamyl transpeptidase), cholesterol, or lipoproteins (ie. HDL) were observed for employees with PFOS concentrations less than 6 ppm (95% of serum PFOS concentrations). Employees

with PFOS concentrations greater than 6 ppm had inconclusive results, with respect to other lesser exposure categories, because of the lack of Antwerp subjects in that category during 1997. Another report examined Alabama workers with at least one year of cumulative employment at a perfluorooctanesulfonyl fluoride manufacturing plant (PFOS and its related precursors are all derived from perfluorooctanesulfonyl fluoride) (n=2083, 83% males), but effects were inconclusive. [6] Despite 65 deaths reported in the high exposure cohort (based on duties in the plant), overall mortality rates were lower for the entire cohort than in the general population of Alabama (standard mortality ratio (SMR)=0.63, 95% CI=0.53-0.74). Additionally, three deaths from bladder cancer were reported among workers who had high exposures (SMR=12.77, 95%CI=2.63-37.35) , but it was uncertain if these occurrences were related to fluorochemical exposure, as these cases were from workers in non-production jobs such as maintenance, incinerator, and wastewater treatment plant operations. Finally, no significantly increased standardized mortality ratio for men (n=2788, SMR= 0.77, 95%CI=0.69-0.86) or women (n=749, SMR=0.75, 95%CI=0.56-0.99) was seen with perfluorooctanoic acid (PFOA) manufacturing employment between 1947 to 1983.[7] However, the risk of prostate cancer mortality was elevated about 3.3 fold (95%CI=1.02-10.6) amongst those who worked for 10 years in comparison to those who were not employed in the PFOA plant. The authors concluded that since there were only four deaths due to prostate cancer among exposed workers in comparison to 6 overall (i.e. including those with no employment in PFOA plant), these results should be interpreted carefully.[7]

Non-occupational studies examining human health impacts of PFAs are few, and reports have focused mostly on birth weight effects. However, results have varied. For example, small negative associations were found with increasing PFOS (per ln-unit: slope: -69 g, 95% CI: -149 to 10) and PFOA (slope : -104 g, 95% CI, -213 to 5) concentrations and birth weight, after adjusting for confounding factors (n=293) in a study by Apelberg et al.(2007).[8] Washino *et al.* reported that PFOS concentrations were negatively associated (per log10 unit: slope: -148.8 g; 95% CI: -297.0 to -0.5 g) with low birth weight in a hospital based prospective study (n= 428 women).[9] However, Fei *et al..*,[10] in a study within the Danish national birth cohort reported that maternal PFOS concentrations were not associated with fetal growth indicators, but that PFOA showed inverse associations with PFOA concentrations and birth weight (n=1400, slope adjusted for gestastional age at birth (days): -10.63 g; 95% CI: -20.79 to -0.47 g per ng/mL increased PFOA). Fei et al.also showed association with PFOA and birth length (decreased by 0.069 cm, 95% CI: 0.024 to 0.113) and abdominal circumference (decreased by 0.059 cm, 95% CI: 0.012 to 0.106) per unit ng/mL increase of PFOA.[11] The first study in Canada showed no association between birth weight and serum PFAs at delivery (n=101, PFOS: slope=0.000853, p=0.7, PFOA: slope=0.000171, p=0.7, ), or in cord blood serum (n=105, PFOS: slope=0.0086, p=0.2, , PFOA: slope=0.000110, p=0.763, ).[12] Another recent Canadian study by Hamm et al.similarly, did not show any relationship between perfluorinated acid exposure and low birth weight.[13] Here, maternal exposure to PFOA, PFOS, and perfluorohexane

sulfonate (PFHxS) did not have an influence on the risk for delivering a small for gestational age (SGA) infant: PFOA relative risk (RR): 1.15 (95% CI 0.49-2.69), PFHxS: RR 1.54 (95% CI 0.91-2.63), and PFOS: RR 0.80 (95% CI 0.50-1.28) (per log unit). Other parameters examined for associations with PFAs include fecundity.[14] Here, Fei et al.(2009) suggested that increasing PFOS and PFOA concentrations in pregnant women, measured at 4-14 weeks pregnancy from the Danish National Birth Cohort (n=1240), were related to decreased fecundity.[14] More specifically, longer reported time to pregnancy (divided into five categories of <1, 1-2, 3-5, 6-12 and >12 months) was associated with higher concentrations of PFOA and PFOS ($p<0.001$). Additionally, adjusted odds ratio (for maternal age at delivery, parity, pre-pregnancy body mass index, maternal socio-occupational status, paternal education, paternal age, and alcohol consumption before pregnancy) of infertility increased by 70-134% and 50-154% in the higher three quartiles of PFOS and PFOA when compared with women in the lowest quartile.

Overall, human health effects with PFAs have not been widely studied, and there are currently no reviews on human health effects of PFAs. Animal studies however, have been numerous. The toxic effects and toxic potency of PFAs differs by perfluorinated chain-length and acid functional group (ie. carboxylate versus sulfonate),[1] and the existing studies have focused largely on PFOS and PFOA. Studies with PFOS have identified the following consistent physiological effects: total serum cholesterol reduction,[15] hepatic peroxisome proliferation,[16-19]

general hepatotoxicity [15, 18, 20, 21] and disruption of thyroid hormone homeostasis.[15, 22-28] In 1988, Gutshall et al.[29] and Langley et al.[30] were the first to report evidence for disruption of thyroid hormone homeostasis by PFAs, whereby a single dose of perfluorodecanoic acid (PFDA) produced a reduction in total serum triiodothyronine (T3) and thyroxine (T4) in rats. The total T4 was reduced in male Wistar rats by as much as 35% after a single high intraperitoneal dose of PFDA (75 mg/kg).[30] A later study by Gutshall et al.[31] demonstrated that PFDA displaced radiolabeled T4 from rat albumin with an affinity comparable to that of endogenous T3. This effect was later consistently observed *in vivo* with related PFAs, PFOA and PFOS, in cynomolgus monkeys, rats, and mice.[15, 23] An interesting effect observed in most animal studies is that the thyroid hormone disturbance caused by PFAs usually occurred without a compensatory response by thyroid stimulating hormone (TSH). Thus, the effects of PFA exposure in animals are consistent with hypothyroxinemia, a condition whereby TSH concentrations are normal, despite relatively low free T4 concentrations.

Given the evidence for disruption of thyroid hormone homeostasis by PFAs at high concentrations, a relevant public health question is thus whether human exposure to background concentrations of PFAs is a risk factor for maternal hypothyroxinemia. Maternal hypothyroxinemia can have adverse impacts on development of offspring. In humans these effects have been investigated in children born to hypothyroxinemic mothers at 3 weeks, [32] 1 year, 2 years, [33] and 3 years of age.[34] From these studies, evidence exists for decreased

neuropsychodevelopment (ie. delayed mental and motor function accessed by Bayley Scales of Infant Development) in children of mothers who had the lowest free T4 concentrations early in pregnancy. No human studies have specifically examined the influence of PFAs on hypothyroxinemia, but there have been studies examining the effect of PFAs on thyroid hormones and other related conditions. Emmett et al.[35] examined the relationship between PFA exposure, thyroid disease, and TSH by studying a community located in Little Hocking, OH (n=371) with considerable environmental exposure to PFOA from a contaminated water supply (median serum PFOA concentration: 354 ng/mL, interquartile range: 184-571 ng/mL). No statistically significant associations were found between PFOA and TSH concentrations ( p=0.4) and although individuals with thyroid disease (n=40) had lower PFOA concentrations (387 ng/mL) than those without thyroid disease (451 ng/mL), this was not statistically significant ( p=0.3). Inoue et al.[36] found no correlations between PFOS exposure and either TSH or free T4 concentrations (correlation coefficients not given) in a very small group of pregnant women (n=15, age 17-37) who enrolled in the study at the Sapporo Toho hospital in Japan. In a slightly larger study, Peck et al.[37] also reported no associations between free T4 and PFOS in pregnant women (n=42), but did show an apparent increase of 0.7 mU/L TSH with higher PFOS concentrations. In the most recent study to date, Dallaire et al.(2009) examined 41 contaminants, including PFOS, with total T3, free T4, and TSH concentrations in Inuit adults (n=623) from Nunavik. [38] Curiously, PFOS was found to be negatively associated with TSH: unadjusted slope (β)= -0.017 µIU/ng, adjusted (for age, sex, body mass

index, plasma lipids, cigarette consumption and education) β = -0.102 μIU/ng ($p \leq 0.05$) and total T3: unadjusted β= -0.030 pmol/ng ($p < 0.001$), adjusted β= -0.017 pmol/ng ($p \leq 0.05$), but positively associated with free T4: unadjusted β= 0.004fmol/ng, adjusted β= 0.014 fmol/ng ($p \leq 0.05$).[38]

The current study uses a case-control design and is the first designed specifically to examine the risk of maternal hypothyroxinemia with PFA exposure in Edmonton, AB, Canada.

## 3. 2. Materials and Methods

### 3. 2. 1. Human Serum Samples

Maternal sera samples (n=974) were taken from an archive of samples from women who elected to undergo second trimester (15-16 weeks) prenatal "triple screens" for trisomy 18, Down's Syndrome, and open spina bifida. These samples are normally discarded, but we obtained ethics approval from the University of Alberta Health Research Ethics Board for use of leftover volumes for the current work. All samples were taken from women in the former Capital Health administrative region of Alberta, which encompasses the city Edmonton. Samples were collected between December 15, 2005 and June 22, 2006 from women who were ≥ 18 years of age and who delivered at ≥ 22 weeks gestation to live singletons without any evidence of malformations. To avoid introducing potential bias from high risk pregnancies, we limited the samples of serum to

those women who were referred by a physician that made at least 8 total recommendations for the triple screen over the study period. All sera were stored at -80°C until analysis of thyroid hormones and PFAs.

### 3. 2. 2. Chemicals

Formic acid (50 %, HPLC grade), PFOS, and PFHxS (>98% purity) were acquired from Fluka (Oakville, ON, Canada). PFHxS and PFOS were sold as potassium salts and were composed of a mixture of linear and branched isomers. PFOA was purchased from Sigma-Aldrich (Oakville, ON, Canada) and is predominately a linear isomer standard. Methanol (HPLC grade) and ammonium hydroxide (Optima grade) were obtained from Fisher Scientific (Ottawa, ON, Canada). Linear isotopically labeled linear isomers of $^{13}$C- PFOA and $^{13}$C-PFOS were obtained from Wellington Laboratories (Guelph, ON, Canada) for use as internal standards. Reagent grade water was prepared by a Millipore Milli-Q system (Bedford, MA, USA). All reagents were stored at room temperature and made fresh every 2 weeks.

### 3. 2. 3. Selection of Cases and Controls

This investigation was a matched case-control study and was limited to singleton births. All 974 samples were analyzed for TSH and free T4. Among these, the hypothyroxinemic cases were defined as those patients exhibiting normal TSH

concentrations with no evidence of hyperthyroidism (0.15 to ≤4 mU/L) and free T4 in the lowest $10^{th}$ percentile (7.4 pmol/L). Controls were also defined as having normal TSH concentrations, but were selected from those samples having free T4 concentrations between the $50^{th}$ and $90^{th}$ percentiles (12 to 14.1 pmol/L). Each case was matched between one and three controls based on physician (n=29) and maternal age (± 3 years). Overall, 96 cases and 175 controls were selected (703 subjects did not meet eligibility). The ratio of controls to cases was not exactly 2 because some cases had too few suitable controls. Thirty three subjects could not be matched according to age criteria (± 3years). Among cases who had matching controls within referring physician, 24% of the cases had one control ($n_{cases}$= 23), 70% had two controls ($n_{cases}$= 67), and 6% had three controls ($n_{cases}$= 6).

### 3. 2. 4. Thyroid Hormone Analysis

Serum analysis for TSH and free T4 was performed according to standard laboratory procedures on a commercial instrument that used a competitive immunoassay with direct chemiluminescent technology and an automated analyzer (Bayer Advia Centaur). Here, acridinium ester-labeled T4 and endogenous free T4 in the maternal sera compete for polyclonal rabbit anti-T4 antibody, thus creating an inverse relationship between the amount of light units detected and serum free T4 concentrations. Precision was tested using three controls (low, medium, and high concentrations) for free T4 and TSH. All three

controls were tested twice a day and run for 15 days consecutively. The precision of TSH concentrations for the Advia Centaur instrument at the lowest concentrations was approximately 10%, while concentrations above this showed coefficients of variation of about 2.7% when tested with 0.34, 4, and 25 mU/L TSH. Coefficients of variation for free T4 varied from 3.0 to 4.0% at all concentrations tested (7, 28 and 60 pmol/L).

### 3. 2. 5. Serum Preparation and Extraction for PFA Analysis

Solid phase extraction of serum was performed by a methodology similar to that of Kuklenyik et al..[39] Samples were prepared by adding 5 ng of internal standard ($^{13}$C-PFOS, $^{13}$C-PFOA) and 3 mL of 0.1 M formic acid to 0.5 mL of serum in a 15 mL polypropylene tube. Samples were then sonicated for 20 min. Oasis-HLB columns (200 mg, 6cc) (Waters Corp, Taunton, Massachusetts, USA) were pre-conditioned with HPLC grade methanol (6 mL) and 0.1 M formic acid (6 mL). Serum samples were loaded quantitatively onto the cartridge using 1 mL of 0.1 M formic acid to rinse the polypropylene tube. The cartridges were then washed with 6 mL of 50% 0.1 M formic acid in methanol, followed by 3 mL of 1% ammonium hydroxide in water, and subsequently vented with air for 5 min. Analytes were eluted with 6 mL of HPLC grade methanol into clean 15 mL polypropylene tubes, and the cartridges were vented with air for another 10 min to collect all eluent. The eluent was concentrated under a stream of dry nitrogen to 0.5 mL and brought up to volume, if necessary, with methanol. The samples were centrifuged

for 10 min at 4000 rpm to remove suspended particles, and 250 μL of supernatant was gently transferred to 0.75 mL polypropylene HPLC vials, vortexed for 1 min, and stored at 4°C until analysis by HPLC-MS/MS.

### 3. 2. 6. Analysis by HPLC-MS/MS

PFA analysis was conducted with an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA, USA) coupled to a hybrid triple-quadrupole linear ion trap mass spectrometer (4000 QTRAP, MDS Sciex, Concord, ON, Canada) equipped with an electrospray interface operating in negative ion mode. Chromatographic separation was accomplished by injecting 10 μL of sample extract onto a Zorbax C8 column (3.5 μM, 2.1 mm x 5 cm, Agilent Technologies, Palo Alto, CA, USA). A reverse phase Zorbax C18 column (5μm, 4.6mm x 15cm, Agilent Technologies, Palo Alto, CA, USA) was placed upstream of the injector to trap and separate instrumental PFA contamination. Gradient elution consisted of 10 mM ammonium acetate (pH = 6.4) as solvent A, and 100% methanol as solvent B at a constant flow rate of 0.18 mL/min. Starting conditions were 80% A, 20% B. The gradient was ramped linearly to 30% A and 70% B by 1 min, and 100% B by 8 min, followed by an 11.5 min hold and returning to starting conditions by 21 min. Initial conditions were held for a further 19 min to allow for column equilibration, resulting in a total run time of 40 min. Infusion of PFOA, PFHxS and PFOS native standard was used to optimize instrumental parameters including curtain gas (20 L/min), collision gas (12 L/min), ion spray voltage (-4500 V), entrance

potential (-10 V), collision exit potential (-15 V), source temperature (350°C) and ion source gas temperature (50°C). Multiple reaction monitoring (MRM) was used to monitor multiple MS/MS transitions per analyte, and PFHxS was quantified in its interference free transition, m/z 399→119, [31] as recommended in Chapter 2.

### 3. 2. 7. Quality Control

Cases and controls were analyzed for perfluorinated acids blind, revealed at time of epidemiological analysis, and were furthermore divided into batches of approximately 16 per set for daily analysis of PFAs. Each set contained a water blank, and a paired sample of spiked (50 ppb of mixed standard) and unspiked serum for spike/recovery calculations. Limits of detection (LOD) were defined in this work as 0.25 ng/mL, corresponding to the lowest standard in our standard curves. All samples were blank subtracted, and average blank concentrations for PFOA, PFOS and PFHxS were below LOD. Percent recoveries from spiked sera were used as an additional means of quality assurance with each batch of samples. Overall accuracy (mean of 16 sets) and precision (± standard deviation) were 99.8±15.7 for PFOA, 102±25.2 for PFHxS, and 91.1±13.9 for PFOS. Concentrations in samples were not adjusted for the within-batch percent recovery because we found no correlation between within-batch recovery and average concentrations in the maternal serum samples (PFOA: r=-0.08, p=0.2, PFHxS: r=0.1, p=0.1, PFOS: r=0.08, p=0.2). Furthermore, perfluorinated acid

concentrations in each individual sample were quantified with internal standards, thus correction based on the spike-recovery samples should not be necessary.

### 3. 2. 8. Statistical Analysis

Statistical analysis was performed using STATA software (Stata Version 8.0 StataCorp, College Station, TX). Molar PFA concentrations were natural logarithm transformed for statistical analysis and concentrations below the LOD were substituted with half the value of the LOD (ie. 0.125 ng/mL). Odds ratios (OR) and 95% confidence intervals (CI) were determined using conditional logistic regression. Potential confounders were included in the adjusted models (maternal age, maternal weight, maternal race, and gestational age). Student's t-tests were used to calculate differences in means between cases and controls for maternal age, maternal weight, and gestational age. Chi-square tests were used to analyze for potential differences between cases and controls in terms of maternal race. Relationships between continuous variables (gestational age at blood draw, maternal age and maternal weight) and PFA concentrations were determined by examining correlation coefficients.

For statistical analyses, gestational age, and maternal age were dichotomized into categories of "low" and "high" based on $50^{th}$ percentile cutoffs for each variable. Maternal race data was initially divided into 8 categories (0=Caucasian; 1=Asian;2=Hispanic; 3=Black; 4=Semitic; 5=Filipino; 6=First

Nation;7=Oriental; 9=Unknown) but for statistical analysis was collapsed these into two major groups: 0= Caucasian and 1= other. For all conditional logistic regression models, categorized forms of gestational age, maternal age, maternal race, and maternal weight were used.

## 3. 3. Results

### 3. 3. 1. Matching Factors and Other Variables

Maternal age of the subjects ranged from 20.1 to 45.1 years (mean: 31.3 ± 4.1 (standard deviation)). (Table 3-1) Hypothyroxinemic cases had a similar but slightly greater mean maternal age (31.6 ± 4.4 yrs) than controls (31.1 ± 3.9 yrs). Gestational age at time of blood draw (mean: 112.7 ± 3.2 days) ranged from 105 to 118 days, whereby cases had a similar but slightly higher mean gestational age than controls (113.2 ± 3.1 days and 112.4 ± 3.2 days, respectively). (Table 3-1) Maternal weight ranged from 88 to 347 pounds (mean: 154.0 ± 35.6 lbs). (Table 3-1) Cases were slightly heavier than controls (mean: 159.3 ± 38.0 and 151.1 ± 33.9 lbs for cases and controls, respectively). For both cases and controls, race was predominantly Caucasian (cases: 76%, controls: 66%), and among cases there were neither Semitic nor Filipino participants. (Table 3-2) A Chi-square test on maternal race showed that there was no statistically significant difference between cases and controls (p=0.5), thus for all statistical analyses race was dichotomized as either Caucasian, or other.

**Table 3-1 Gestational age at test, maternal age, maternal weight, free T4, and TSH concentrations (96 cases and 175 controls).**

| | Mean | 25th percentile | 50th percentile | 75th percentile | Min | Max | p-value for difference in means* |
|---|---|---|---|---|---|---|---|
| Gestational age at test (days) | | | | | | | |
| Total | 113 | 111 | 113 | 115 | 105 | 118 | 0.04 |
| Cases | 113 | 111 | 113 | 116 | 105 | 118 | |
| Controls | 112 | 111 | 113 | 115 | 105 | 118 | |
| Maternal age (years) | | | | | | | |
| Total | 31.3 | 28.3 | 31.2 | 34.0 | 20.1 | 45.1 | 0.3 |
| Cases | 31.6 | 28.3 | 31.4 | 34.3 | 20.1 | 45.1 | |
| Controls | 31.0 | 28.2 | 31.0 | 33.9 | 23.2 | 41.1 | |
| Maternal weight (pounds) | | | | | | | |
| Total | 154 | 128 | 148 | 173 | 88 | 347 | 0.07 |
| Cases | 159 | 132 | 153 | 178 | 105 | 347 | |
| Controls | 151 | 126 | 145 | 170 | 88 | 281 | |
| free T4 concentrations (pmol/L) | | | | | | | |
| Total | 11.1 | 8.3 | 12.0 | 13.2 | 5.8 | 15.0 | <0.001 |
| Cases | 7.7 | 7.3 | 7.9 | 8.4 | 5.8 | 8.7 | |
| Controls | 12.9 | 12.1 | 12.8 | 13.6 | 11.6 | 15.0 | |
| TSH concentrations (mU/L) | | | | | | | |
| Total | 0.97 | 0.56 | 0.86 | 1.19 | 0.16 | 3.26 | <0.001 |
| Cases | 0.69 | 0.43 | 0.65 | 0.89 | 0.16 | 1.90 | |
| Controls | 1.13 | 0.69 | 0.99 | 1.40 | 0.17 | 3.26 | |

*difference in means calculated with Student's t-test

**Table 3-2 Breakdown of maternal race among case and control samples**

| | Total (n) | Cases (n) | Controls (n) |
|---|---|---|---|
| **Caucasian** | 189 | 73 | 116 |
| **Asian** | 29 | 8 | 21 |
| **Hispanic** | 2 | 1 | 1 |
| **Black** | 4 | 1 | 3 |
| **Semitic** | 2 | 0 | 2 |
| **Filipino** | 5 | 0 | 5 |
| **First Nation** | 4 | 2 | 2 |
| **Oriental** | 16 | 4 | 12 |
| **Unknown** | 20 | 7 | 13 |
| **Total** | 271 | 96 | 175 |

### 3. 3. 2. Associations between Gestational Age at Blood Draw, Maternal Age, Race, and Weight, and Hypothyroxinemia.

Despite several of the variables showing possible association with hypothyroxinemia, no association was significant and CIs were relatively wide (Table 3-3). However, when gestational age at test was dichotomized, the high category (≥113 days) showed a 69% increased adjusted OR for hypothyroxinemia. Older mothers showed a 38% increase in adjusted OR for hypothyroxinemia compared to their younger counterparts when subjects were divided into categories of low and high age. Similarly, dichotomized maternal weight (low and high) showed that the high category (≥154 pounds) had a 49% increased adjusted OR for hypothyroxinemia in comparison to the low category (<154 pounds), but again the estimate of the effect was not precise. Non -Caucasians had a decreased OR for hypothyroxinemia (0.58 adjusted ORs) compared to Caucasians.

**Table 3-3 Odds ratios (crude and adjusted) for hypothyroxinemia and gestational age at blood draw, maternal age, maternal race, and maternal weight using conditional logistic regression.**

| *Variable* | | | | *Univariate Analysis* | | *Multivariate Analysis* | |
|---|---|---|---|---|---|---|---|
| | | ***N cases*** | ***N controls*** | ***OR*** | ***95 % CI*** | ***OR*** | 95 % CI |
| Gestational Age at blood draw | <113 days | 35 | 86 | 1 | -- | 1 | -- |
| | ≥113 days | 61 | 89 | 1.66 | 0.99-2.78 | 1.69[a] | 0.99-2.90[a] |
| Maternal Age | <31.3 years | 44 | 89 | 1 | -- | 1 | -- |
| | ≥ 31.3 years | 52 | 86 | 1.59 | 0.74-3.42 | 1.38[b] | 0.63-3.02[b] |
| Maternal Race | Caucasian | 73 | 116 | 1 | -- | 1 | -- |
| | non-Caucasian | 23 | 59 | 0.57 | 0.30- 1.06 | 0.58[c] | 0.30-1.10[c] |
| Maternal Weight | <148 pounds | 42 | 92 | 1 | -- | 1 | -- |
| | ≥148 pounds | 54 | 83 | 1.57 | 0.91-2.72 | 1.49[d] | 0.85-2.61[d] |

[a] adjusted for maternal age, maternal race, and maternal weight.

[b] adjusted for gestational age at draw, maternal race, and maternal weight.

[c] adjusted for gestational age at draw, maternal age, and maternal weight.

[d] adjusted for gestational age at draw, maternal age, and maternal race

### 3. 3. 3. PFA Concentrations

Logarithmically transformed molar data for PFAs were approximately normally distributed (Figure 3-1), thus PFA concentrations were transformed to logarithms of their molar concentrations for statistical analysis. There were no statistically significant differences between mean concentrations of cases and controls for PFOA (p= 0.6), PFHxS (p=0.5) or PFOS (p=0.4). (Table 3-4) For the total population, the geometric means for PFOA, PFHxS, and PFOS were 3.25, 2.68, and 14.8 mmol/L, respectively, with geometric standard deviation (GSD) of 2.85, 2.97, and 2.04, respectively. (Table 3-4).

Mean concentrations of PFAs here were similar to those reported in pregnant women from a province-wide biomonitoring project (PFOA: 2.6 ng/g, PFHxS: 2.1 ng/g, PFOS: 7.4 ng/g) conducted in Alberta in the same time-frame as the current study.[40] Another published report for pooled samples of pregnant women in the Edmonton region, collected in 2006, also showed comparable mean concentrations of all three PFAs: PFOA (4.0 ng/g), PFHxS (1.2 ng/g), and PFOS (9.1 ng/g).[41] In comparison, the first Canadian study to investigate the association between low birth weight and PFA exposure reported similar PFOA (2.54 ± 1.65 ng/mL) in pregnant women (24-28 weeks) but higher PFHxS (4.13 ± 11.43 ng/mL) and PFOS (18.31 ± 10.95 ng/mL) concentrations .[12] This first Canadian study was conducted in 2004/2005, so it is feasible that the lower PFOS concentrations found in our study reflect declining PFA concentrations due to

national and international regulatory policies. However, spatial variation across Canada cannot be ruled out, since samples in the first Canadian study were collected from Hamilton, Ontario.[12] Additionally, as observed in previous studies with humans,[42] all three PFAs were positively correlated with each other. The strength of the correlations were very similar amongst all comparisons. (Figure 3-2)

**Table 3-4 PFA concentrations among cases (96) and controls (175)**

| | N<0.25 (limit of detection) | Standard Deviation | Mean ($log_e$ mmol/L) | $25^{th}$ percentile ($log_e$ mmol/L) | $50^{th}$ percentile ($log_e$ mmol/L) | $75^{th}$ percentile ($log_e$ mmol/L) | Min ($log_e$ mmol/L) | Max ($log_e$ mmol/L) | p- value for difference in means* |
|---|---|---|---|---|---|---|---|---|---|
| Perfluorooctanoic acid (PFOA) | | | | | | | | | |
| Total | 24 | 1.05 | 1.18 | 0.69 | 1.31 | 1.84 | -1.20 | 3.74 | 0.6 |
| Cases | 11 | 1.13 | 1.13 | 0.59 | 1.37 | 1.84 | -1.20 | 3.28 | |
| Controls | 13 | 1.00 | 1.20 | 0.77 | 1.28 | 1.82 | -1.20 | 3.74 | |
| Perfluorooctane sulfonate (PFOS) | | | | | | | | | |
| Total | 1 | 0.71 | 2.69 | 2.34 | 2.76 | 3.16 | -1.39 | 4.25 | 0.5 |
| Cases | 1 | 0.73 | 2.65 | 2.34 | 2.74 | 3.13 | -1.39 | 4.11 | |
| Controls | 0 | 0.70 | 2.72 | 2.34 | 2.80 | 3.17 | 0.18 | 4.25 | |
| Perfluorohexane sulfonate (PFHxS) | | | | | | | | | |
| Total | 19 | 1.09 | 0.99 | 0.30 | 0.89 | 1.64 | -1.16 | 4.67 | 0.4 |
| Cases | 6 | 1.14 | 1.05 | 0.38 | 0.90 | 1.74 | -1.16 | 4.67 | |
| Controls | 13 | 1.06 | 0.95 | 0.30 | 0.85 | 1.59 | -1.16 | 4.02 | |

*** Mean differences calculated by Student's t-test.**

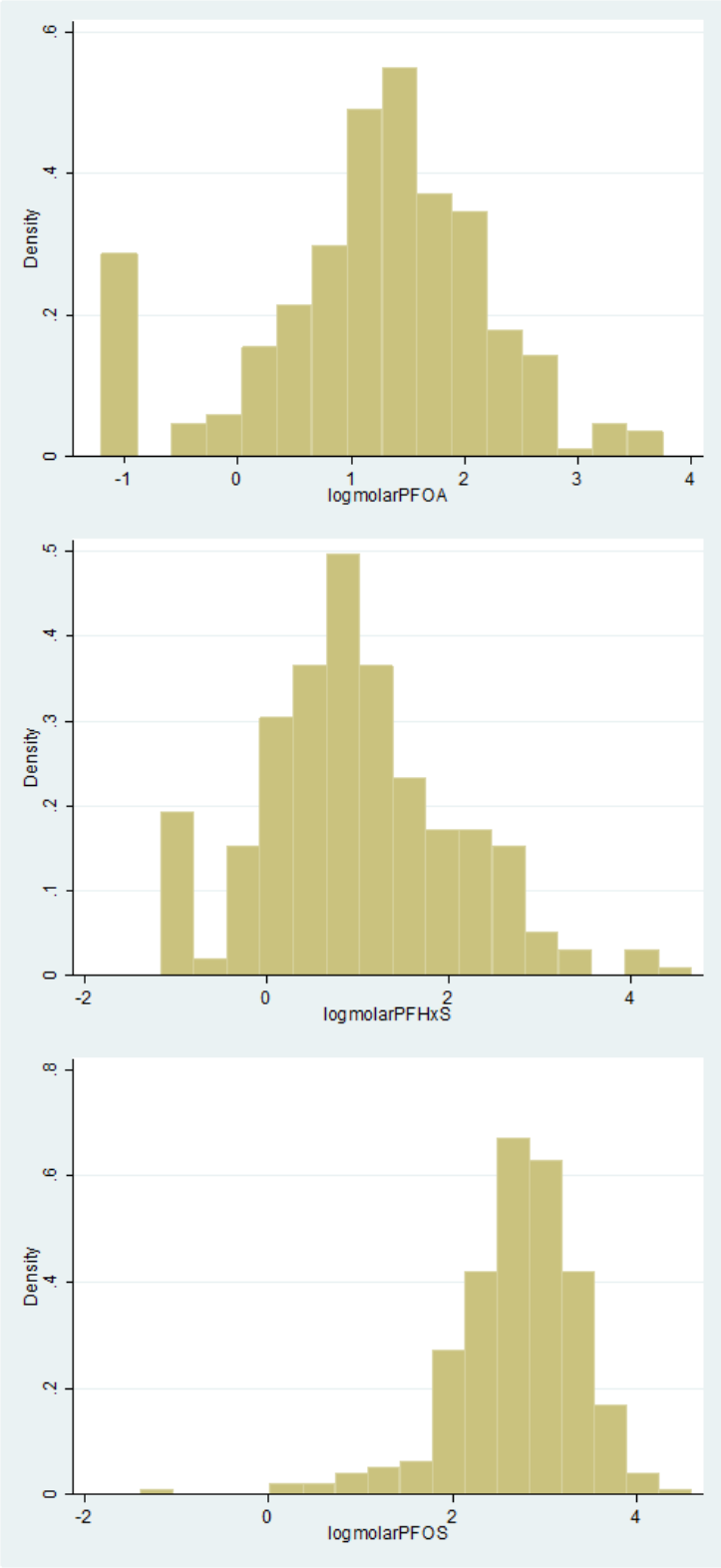


**Figure 3-1 Density histograms of log molar PFOA, log molar PFHxS, and log molar PFOS ($log_e$ (mmol/L)) concentrations amongst study population.**

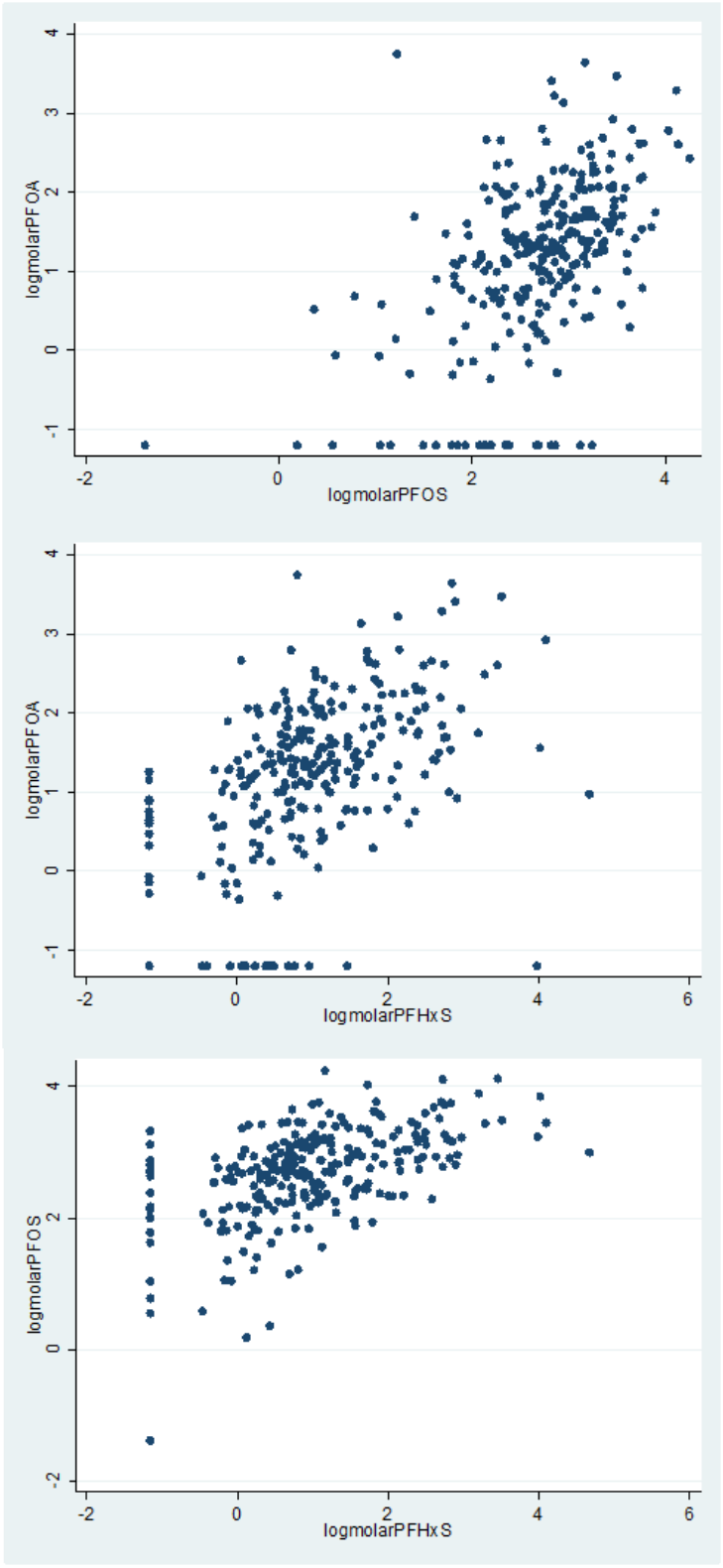


**Figure 3-2 Scatterplot of a) PFOA versus PFOS ($log_e$(mmol/L)): r= 0.5, p< 0.005, b) PFOA versus PFHxS ($log_e$(mmol/L)): r= 0.5, p< 0.005, c) PFOS versus PFHxS ($log_e$(mmol/L): r= 0.5, p<0.005.**

Gestational age at blood draw, maternal age, and maternal weight were not associated with PFA concentrations, as evidenced by a lack of significant correlations ($p>0.05$) between these variables and blood PFA concentrations. (Figures 3-3 to 3-5) More specifically, there were no significant correlations between gestational age and log molar PFOA ($r=0.02$, $p=0.7$), PFHxS ($r=-0.03$, $p=0.6$) or PFOS ($r=0.002$, $p\geq0.9$) concentrations. No significant correlations were found between maternal age and log molar PFOA ($r=0.01$, $p=0.9$), PFHxS ($r=-0.02$, $p=0.8$), and PFOS ($r=0.04$, $p=0.5$). Lastly, maternal weight did not show any relationship with log molar PFOA ($r=0.1$, $p=0.1$) PFHxS ($r=0.08$, $p=0.2$) nor PFOS ($r=0.09$, $p=0.1$) concentrations. Finally, there were no large changes in precision or point estimates of ORs in univariate versus multivariate analyses (appropriately adjusted per variable for gestational age at blood draw, maternal age, maternal race, or maternal weight and exposure). (Table 3-3)

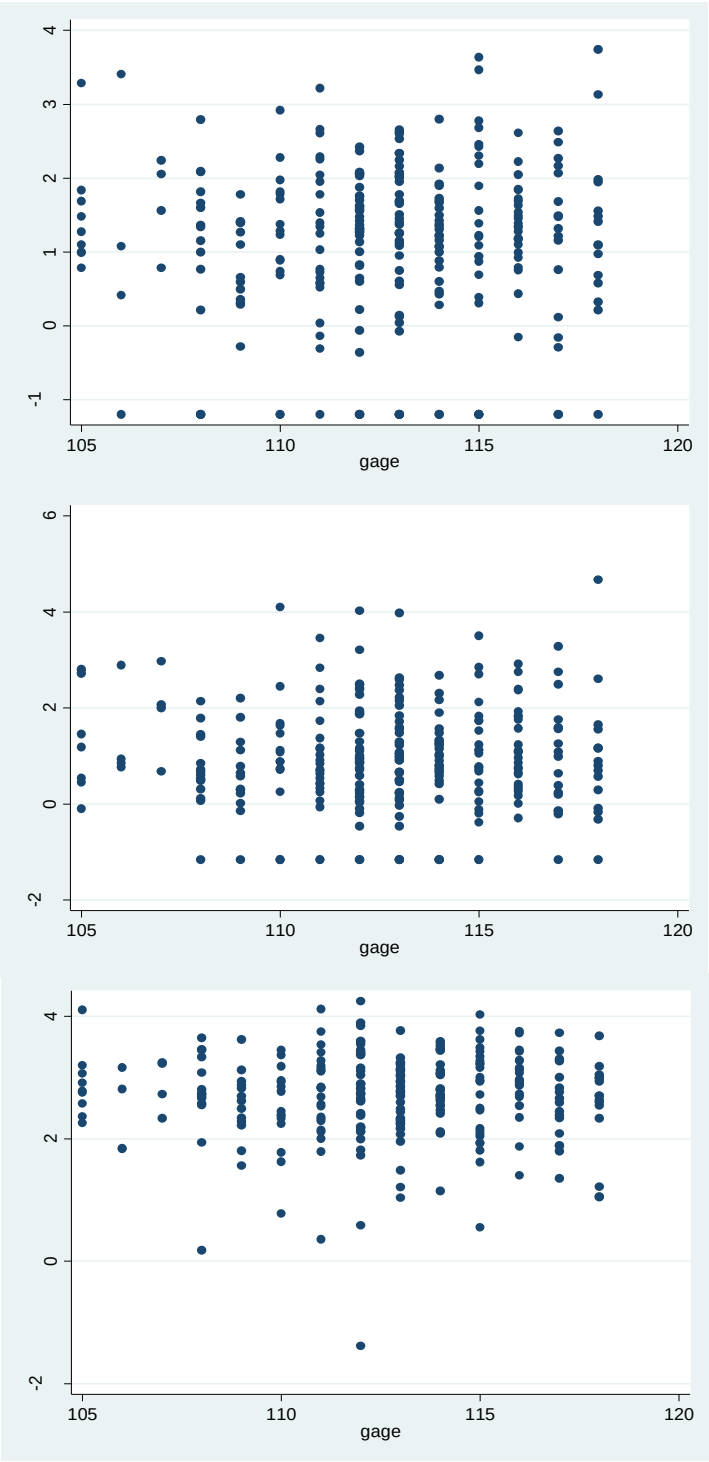


**Figure 3-3 Scatterplot of log molar PFOA, PFHxS, and PFOS ($log_e$ mmoL/L) and gestational age at blood draw ("gage", days).**

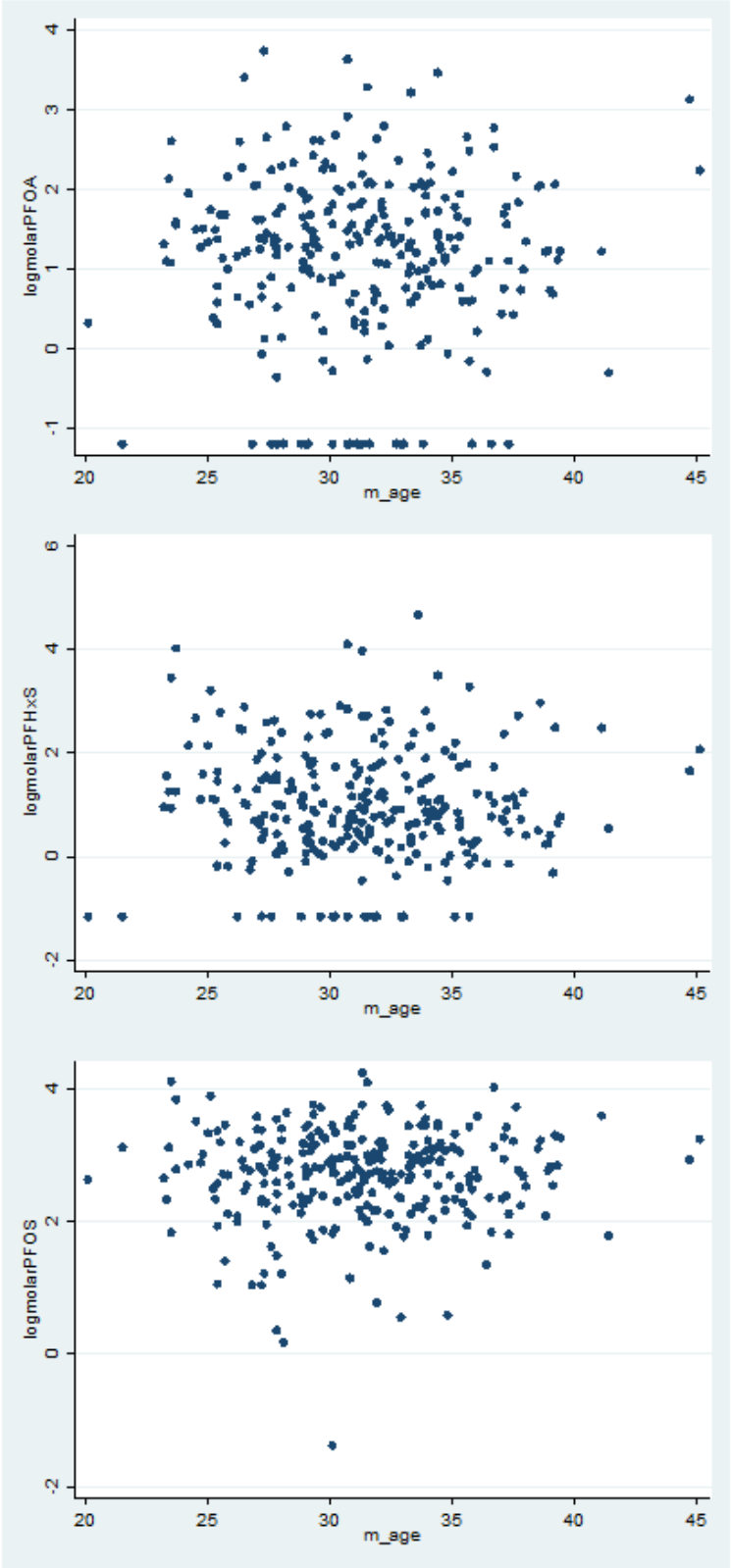


**Figure 3-4 Scatterplot of log molar PFOA, PFHxS, and PFOS ($log_e$ mmoL/L) with maternal age ("m_age", years).**

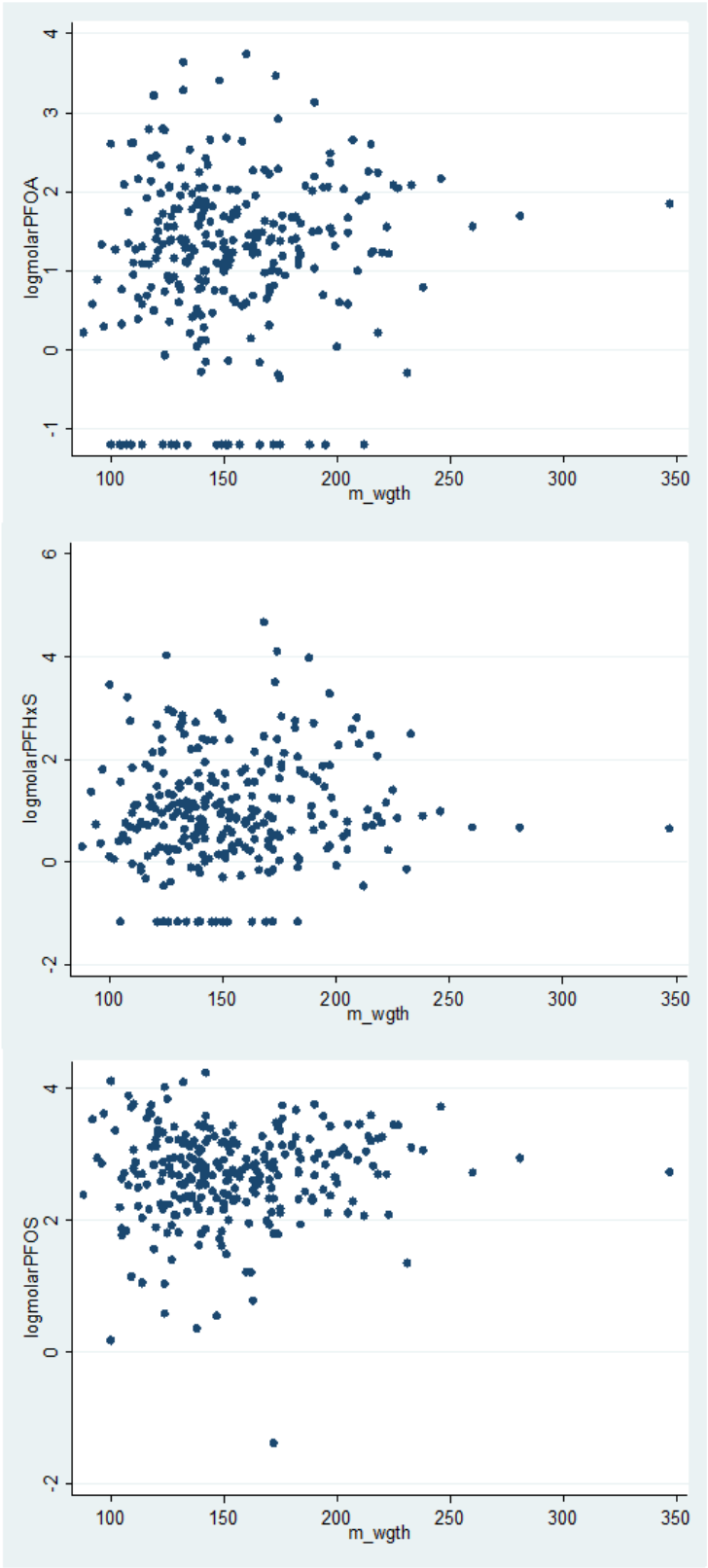


**Figure 3-5 Scatterplot of logmolar PFOA, PFHxS, and PFOS ($log_e$ mmoL/L) with maternal weight ("m_wgth", pounds).**

Interestingly, dichotomizing maternal race did show differences in PFOA ($p<0.05$), PFHxS ($p<0.05$), and PFOS ($p<0.05$) concentrations between groups. In this study, $\log_e$ molar concentrations of PFOA, PFOS, and PFHxS were higher in Caucasians in comparison to non-Caucasians. The nature of these differences was explored by breaking out non-Caucasians into individual races (Table 3-5). In general, Caucasians were among the most highly exposed race to PFOA, PFHxS, and PFOS. For PFOA, Caucasians showed the highest mean concentrations, but were similar to those of Semitics. For PFHxS concentrations, Hispanics actually had highest mean concentrations, but exposure was practically identical to Caucasians and, furthermore, Caucasians had the highest maximum concentration overall. Similarly, Caucasians had the highest mean and maximum PFOS concentrations, but the mean was almost identical to those of Orientals. These trends are similar to the U.S. population, whereby the National Health and Nutrition Examination Surveys (NHANES) showed PFOA, PFOS and PFHxS least square mean concentrations were higher amongst non-Hispanics whites in comparison to non-Hispanic blacks and Mexican Americans.[14]

**Table 3-5 PFA concentrations amongst races**

| | Standard Deviation | Mean ($log_e$mmol/L) | 25$^{th}$ percentile ($log_e$mmol/L) | 50$^{th}$ percentile ($log_e$mmol/L) | 75$^{th}$ percentile ($log_e$mmol/L) | Min ($log_e$mmol/L) | Max ($log_e$mmol/L) | N | % cases | % controls |
|---|---|---|---|---|---|---|---|---|---|---|
| **PFOA** | | | | | | | | | | |
| Caucasian | 0.97 | 1.32 | 0.89 | 1.40 | 1.95 | -1.20 | 3.64 | 189 | 76.0 | 66.3 |
| Asian | 1.11 | 0.90 | 0.22 | 1.00 | 1.60 | -1.20 | 3.75 | 29 | 8.33 | 12 |
| Hispanic | 0.16 | 1.22 | 1.11 | 1.22 | 1.33 | 1.10 | 1.33 | 2 | 1.04 | 0.57 |
| Black | 1.18 | -0.61 | -1.20 | -1.20 | -0.016 | -1.20 | 1.16 | 4 | 1.04 | 1.71 |
| Semitic | 0.97 | 1.29 | 0.60 | 1.29 | 1.98 | 0.60 | 1.98 | 2 | 0 | 1.14 |
| Filipino | 1.20 | 0.89 | 1.09 | 1.30 | 1.40 | -1.19 | 1.88 | 5 | 0 | 2.86 |
| First Nation | 1.35 | 1.06 | -0.016 | -0.98 | 2.14 | -0.36 | 2.66 | 4 | 2.08 | 1.14 |
| Oriental | 1.31 | 0.88 | 0.26 | 0.85 | 1.92 | -1.20 | 2.80 | 16 | 4.17 | 6.86 |
| Unknown | 1.06 | 0.97 | 0.50 | 1.12 | 1.74 | -1.20 | 2.66 | 20 | 7.29 | 7.43 |
| **PFHxS** | | | | | | | | | | |
| Caucasian | 1.08 | 1.20 | 0.50 | 1.11 | 1.88 | -1.16 | 4.67 | 189 | 76.0 | 66.3 |
| Asian | 0.79 | 0.38 | 0.22 | 0.40 | 0.81 | -1.16 | 1.79 | 29 | 8.33 | 12 |
| Hispanic | 0.46 | 1.22 | 0.89 | 1.22 | 1.55 | 0.89 | 1.55 | 2 | 1.04 | 0.57 |
| Black | 0.59 | -0.38 | -0.81 | -0.27 | 0.053 | -1.16 | 0.19 | 4 | 1.04 | 1.71 |
| Semitic | 0.56 | 0.68 | 0.29 | 0.68 | 1.08 | 0.29 | 1.08 | 2 | 0 | 1.14 |
| Filipino | 1.14 | 0.32 | -0.092 | 0.12 | 0.79 | -1.16 | 1.94 | 5 | 0 | 2.86 |
| First Nation | 1.57 | 0.56 | -0.56 | 0.40 | 1.68 | -1.60 | 2.59 | 4 | 2.08 | 1.14 |
| Oriental | 0.88 | 0.68 | 0.26 | 0.84 | 1.12 | -1.16 | 1.85 | 16 | 4.17 | 6.86 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Unknown | 1.10 | 0.70 | 0.060 | 0.78 | 1.24 | -1.16 | 2.79 | 20 | 7.29 | 7.43 |
| **PFOS** | | | | | | | | | | |
| Caucasian | 0.56 | 2.85 | 2.54 | 2.89 | 3.22 | 0.58 | 4.25 | 189 | 76.0 | 66.3 |
| Asian | 1.00 | 2.11 | 1.81 | 2.25 | 2.71 | -1.38 | 3.60 | 29 | 8.33 | 12 |
| Hispanic | 0.19 | 2.48 | 2.34 | 2.48 | 2.62 | 2.34 | 2.62 | 2 | 1.04 | 0.57 |
| Black | 0.72 | 1.40 | 0.80 | 1.48 | 1.99 | 0.56 | 2.08 | 4 | 1.04 | 1.71 |
| Semitic | 0.066 | 2.33 | 2.28 | 2.33 | 2.38 | 2.28 | 2.38 | 2 | 0 | 1.14 |
| Filipino | 0.53 | 2.51 | 2.18 | 2.64 | 2.82 | 1.78 | 3.13 | 5 | 0 | 2.86 |
| First Nation | 0.34 | 2.52 | 2.24 | 2.47 | 2.79 | 2.19 | 2.94 | 4 | 2.08 | 1.14 |
| Oriental | 0.82 | 2.84 | 2.29 | 3.01 | 3.53 | 1.15 | 3.77 | 16 | 4.17 | 6.86 |
| Unknown | 0.79 | 2.33 | 1.99 | 2.53 | 2.85 | 0.36 | 3.42 | 20 | 7.29 | 7.43 |

### 3. 3. 4. PFAs as determinants of hypothyroxinemia

Considering the possible effect of each PFA independently on hypothyroxinemia, unadjusted ORs were calculated. PFHxS showed a higher OR in the crude analysis than PFOA or PFOS (Table 3-6). Adjusting for potential confounders decreased the ORs for all PFAs (Table 3-6) although precision of the OR estimates for each PFA did not differ dramatically between the crude and adjusted analyses.

**Table 3-6 Conditional logistic regression for each PFA separately ($log_e$ molar concentration, continuous metric); OR=odds ratio; CI= confidence interval**

| ***PFA*** | ***Crude analysis*** | | ***Adjusted analysis**** | |
|---|---|---|---|---|
| | ***OR*** | ***95 % CI*** | ***OR*** | **95% CI** |
| PFOA | 0.94 | 0.74- 1.18 | 0.87 | 0.68-1.12 |
| PFOS | 0.88 | 0.63- 1.24 | 0.77 | 0.53-1.12 |
| PFHxS | 1.12 | 0.89- 1.41 | 1.06 | 0.83-1.36 |

* adjusted for maternal age, maternal weight, maternal race, and gestational age at blood collection

Given that the concentrations of the three PFAs examined here were correlated with each other (Figure 3-2), the models were re-run with all three PFAs forced into the model (i.e. each PFA was controlled for the other two). Only exposure to PFHxS was associated with an elevated (~30%) odds of hypothyroxinemia (Table

3-7) when controlled for the other two compounds, and this association approached statistical significance. OR point estimates for PFOS and PFOA suggested protective effects (e.g. OR<1.0), but were also not statistically significant (p>>0.05). Adjusting for maternal age, maternal weight, maternal race, and gestational age at blood collection, all ORs decreased (Table 3-8), suggested some degree of mutual confounding. In an attempt to determine if these effects were from chemical mixture interactions with PFHxS, interactions were also tested between PFOS and PFHxS, as well as between PFOA and PFHxS. Here, two-way and three-way interactions showed imprecise and statistically insignificant associations (p>0.05) (Table 3-8).

**Table 3-7 Conditional logistic regression for three PFA log molar concentrations together in the model. ($log_e$ molar concentration, continuous metric); OR= odds ratio; CI=confidence interval**

| ***PFA*** | ***Crude Analysis*** | | ***Adjusted Analysis**** | |
|---|---|---|---|---|
| | ***OR*** | ***95 % CI*** | ***OR*** | **95% CI** |
| PFOA | 0.90 | 0.67- 1.21 | 0.87 | 0.63-1.19 |
| PFOS | 0.79 | 0.51- 1.22 | 0.73 | 0.46-1.16 |
| PFHxS | 1.29 | 0.96- 1.73 | 1.27 | 0.93-1.72 |

* adjusted for maternal age, maternal weight, maternal race, and gestational age at blood collection

**Table 3-8 Two and three way interactions tested for $log_e$ molar PFOA, PFHxS, and PFOS; OR= odds ratio; CI= confidence interval**

| Variable | Category | Crude | | Adjusted* | |
|---|---|---|---|---|---|
| | | OR | 95% CI | OR | 95% CI |
| PFOA | -- | 0.41 | 0.13-1.32 | 0.40* | 0.12-1.37* |
| PFHxS | -- | 3.69 | 0.94-14.6 | 3.41* | 0.81-14.4* |
| PFOS | -- | 0.82 | 0.46-1.45 | 0.73* | 0.40-1.35* |
| Two way | PFOA and PFOS | 1.33 | 0.86-2.96 | 1.34* | 0.84-2.12 |
| Two way | PFOA and PFHxS | 1.02 | 0.47-2.18 | 0.97* | 0.43-2.18* |
| Two way | PFOS and PFHxS | 0.69 | 0.44-1.10 | 0.71* | 0.44-1.15* |
| Three way | All PFAs | 0.995 | 0.79-1.26 | 1.01* | 0.79-1.29* |
| Gestational age at blood draw | <113 days | -- | -- | 1 | -- |
| | ≥113 days | -- | -- | 1.63 | 0.94-2.86 |
| Maternal age | <31.2 years | -- | -- | 1 | -- |
| | ≥31.2 years | -- | -- | 1.40 | 0.61-3.18 |
| Maternal race | Caucasian | -- | -- | 1 | -- |
| | Non-Caucasian | -- | -- | 0.53 | 0.26-1.09 |
| Maternal weight | >148 pounds | -- | -- | 1 | -- |
| | ≥148 pounds | -- | -- | 1.57 | 0.87-2.83 |

*Adjusted for gestational age at blood draw, maternal age, maternal race, and maternal weight.

The results from the analyses with separate PFAs, and all PFAs together, show indications of over-fitting for the statistical models. For example, separate PFAs showed almost no effect. However, when all three PFAs were included in the model together, the directions of the estimated effects of PFHxS diverged from those of PFOA and PFOS. Additionally, the second model with all PFAs forced together showed a wider 95% CI in comparison to the first model with PFHxS alone. Thus, despite a greater OR for PFHxS and hypothyroxinemia when controlling for PFOA and PFOS, the loss of precision suggests that this model may be unreliable.

Testing the molar sum of PFAs is relevant toxicologically given recent *in vitro* toxicological evidence indicating that all three of the PFAs examined here could possibly produce hypothyroxinemia through interaction with the same receptor (i.e. by displacing thyroxine from serum carrier proteins).[43] However, this resulted in an OR that was not significantly different from the null (OR crude: 0.97, 95% CI: 0.68-1.37 and OR adjusted: 0.83, 95% CI: 0.57-1.23). Thus, there is no evidence that at the exposure concentrations examined here that these PFAs can act additively to cause hypothyroxinemia.

### 3. 3. 5. PFAs as determinants of hypothyroxinemia: Analysis by Tertile

By dividing PFA concentrations into low, medium and high tertiles we were able to further examine for possible non-linearity in the relationships between PFA

exposures and hypothyroxinemia (Table 3-9) that might be expected to occur around toxicological thresholds. Adjusted and unadjusted trends were in the same direction and not statistically significant: positive for PFHxS (unadjusted p-value=0.2, adjusted p-values for trend= 0.4), and negative for PFOA (unadjusted p-trend= 0.95, adjusted p-trend = 0.5) and PFOS (unadjusted p-trend=0.6, adjusted p-trend= 0.4). None of the medium or high exposure categories indicated significantly different ORs from the low group.

**Table 3-9 Division of individual PFA log e molar concentrations into tertiles among low, medium and high categories ($log_e$ mmol/L)**

| *PFA* | *Tertile* | *Exposure group* | *N cases* | *N controls* | *Crude Analysis* | | *Adjusted Analysis** | |
|---|---|---|---|---|---|---|---|---|
| | | | | | *OR* | *95% CI* | *OR* | *95% CI* |
| PFOA | Low | < 0.993 | 35 | 55 | 1 | -- | 1 | -- |
| | Medium | ≥ 0.993 ≤ 1.622 | 26 | 64 | 0.58 | 0.30-1.12 | 0.47 | 0.24-0.93 |
| | High | >1.622 | 35 | 56 | 1.00 | 0.56-1.82 | 0.83 | 0.43-1.58 |
| PFOS | Low | < 2.54 | 33 | 57 | 1 | -- | 1 | -- |
| | Medium | ≥ 2.54, ≤ 3.01 | 34 | 56 | 1.08 | 0.59-1.96 | 0.95 | 0.50-1.78 |
| | High | >3.01 | 29 | 62 | 0.84 | 0.45-1.57 | 0.73 | 0.37-1.41 |
| PFHxS | Low | <0.573 | 28 | 63 | 1 | -- | 1 | -- |
| | Medium | ≥ 0.573, ≤ 1.265 | 32 | 57 | 1.23 | 0.63-2.38 | 1.17 | 0.58-2.35 |
| | High | >1.265 | 36 | 55 | 1.57 | 0.84-2.95 | 1.36 | 0.70-2.68 |

* adjusted for maternal age, maternal weight, maternal race, and gestational age at blood collection

Similarly, dividing the molar sum of PFAs into tertiles of low, medium and high showed minimal trend, if any at all. Adjusted and unadjusted p-value trends were non-significant (unadjusted: 0.96, adjusted: 0.6). (Table 3-10) Lastly, testing for interactions among tertiles was not possible with the unadjusted and adjusted models because of an insufficient numbers of subjects.

**Table 3-10 Division of $\log_e$ molar sum PFA concentrations into tertiles of low, medium and high categories**

| ***Tertile*** | ***Exposure group (log mmol/L)*** | ***N Cases*** | ***N controls*** | ***Crude Analysis*** | | ***Adjusted Analysis**** | |
|---|---|---|---|---|---|---|---|
| | | | | ***OR*** | ***95% CI*** | ***OR*** | ***95% CI*** |
| Low | < 2.92 | 34 | 56 | 1 | -- | 1 | -- |
| Medium | ≥2.92, ≤3.45 | 29 | 61 | 0.79 | 0.42-1.48 | 0.76 | 0.40-1.45 |
| High | >3.45 | 33 | 58 | 0.97 | 0.53-1.80 | 0.84 | 0.44-1.60 |

*Adjusted for gestational age at blood draw, maternal age, maternal race, and maternal weight.

## 3. 4. Discussion

### 3. 4. 1. Associations with Gestational Age at Blood Draw, Maternal Age, Race, and Weight, and Hypothyroxinemia.

To examine the possible effects of gestational age, at time of blood collection, on hypothyroxinemia status, gestational age was dichotomized. The high category of gestational age at blood collection showed increased ORs for hypothyroxinemia (Table 3-3), albeit with a large CI range. This may reflect the magnitude of thyroid hormone change that naturally occurs between 105 to 118 days of gestation [44] and suggests the relative importance of confining the time of blood sampling to a narrow range for studies examining maternal thyroid hormones, and furthermore of adjusting for it in statistical models where appropriate.

Increases in maternal age showed small and insignificant increases in ORs for hypothyroxinemia (Table 3-3). Maternal age has not been well examined with hypothyroxinemia itself, but a recent study showed that older maternal age was associated with lower cord total T4.[45] In the general population however, thyroid hormone concentrations increase with age. [46]

Maternal race was divided into Caucasian and Non-Caucasian categories, and results did not indicate that this division segregated women into groups with

different risk of hypothyroxinemia. We are not aware of previous studies that have examined the incidence of hypothyroxinemia by race, but some studies show that race is associated with hypothyroidism. [47]

Maternal weight was not associated with the risk of hypothyroxinemia in our sample population. Most recently however, Rotondi et al.(2009) showed that obese patients had lower free thyroid hormone concentrations in comparison to controls.[48] Similarly, most studies have demonstrated lower T4 concentrations at higher body weight and BMI levels, yet there have also been reports of higher or similar thyroid hormone concentrations in high BMI individuals relative to controls. [49, 50]

### 3. 4. 2. PFA Exposure and Hypothyroxinemia

Our findings do not support existence of a causal link between PFA exposure and maternal hypothyroxinemia in the studied population. A near statistically significant effect for PFHxS, showing a 30% increase in outcome, is most likely explained by overfitting in the conditional logistic regression model when all three PFAs were included. Collinearity of PFAs was apparent: strong correlations between the independent variables (PFOA, PFHxS, and PFOS) increased the chances of inflating coefficient estimate variances, presumably increasing the "significance" of the regression model when all variables were included. [51] Overfitted models generally produce overly optimistic models.[52]

Although the findings in the current study were not statistically significant, and may have been affected by overfitting, the result for PFHxS is worth further discussion in light of recent *in vitro* toxicology work. More specifically, Weiss et al.[43] examined the binding of PFOA, PFOS, and PFHxS (and other PFAs) to the human thyroid hormone transport protein transthyretin (TTR) in a radioligand binding assay. In humans, approximately 10% of thyroid hormone transport occurs through TTR and it is found in higher concentrations during prenatal and early postnatal periods of animals due to its vital role in central nervous system development. [53] Overall, Weiss et al.found that PFA binding potencies for TTR were 12.5-50 times lower than the natural ligand thyroxine (IC50= 61 nM), but interestingly the most potent PFA for displacement of T4 was PFHxS (717 nM), followed by PFOS (940 nM) and PFOA (949 nM). Thus there is perhaps some plausibility that PFHxS could be the only PFA in our study to have shown an association with hypothyroxinemia, but it is important to consider that the lowest observed affect level (LOAEL) for PFHxS was approximately 19 times higher in the *in vitro* study by Weiss et al.than the mean concentration of the PFA found in the current study (i.e. LOAEL: 39.9 ng/mL, and mean PFHxS: 2.09 ng/mL). Only one of our study subjects had a PFHxS concentration above this threshold: one maternal sample had a PFHxS concentration of 42.5 ng/mL, and interestingly this was a hypothyroxinemic case. Furthermore, the ED-RIA experiments of Weiss et al.was performed in Tris-HCl buffer, not serum, and because PFAs are known to

bind strongly to serum proteins (ie. albumin and liver fatty acid binding protein), the physiologically relevant IC50 for PFAs is probably much higher.

The current study was initiated in 2005, but it is notable that since that time the common observation of decreased free T4 concentrations in animals exposed to PFOS has been argued to be an artifact of analogue analytical methods for free T4. [54] Specifically, it was demonstrated that the ADVIA: Centaur (chemiluminescent) and Coat-a-Count analog (radioimmunoassay) methods for free T4 have a negative bias in the presence of high PFOS concentrations. While Chang et al.did demonstrate this bias *in vivo* in rodents, the animals were only tested up to the end of 24 hours, thus subchronic mechanisms leading to hypothyroxinemia were not definitively ruled out for PFOS. Using a reliable method for T4, equilibrium dialysis-radioimmunoassays (ED-RIA), Luebker et al.reported that subchronic PFOS treatment (at doses of 0.8, 1.0, 1.2, and 2.0 mg/kg/d) in rodents did not create a hypothyroid state when measured at day 6 of lactation, [26] however during lactation many physiological changes occur in thyroid hormone regulation which brings into question whether all previous toxicological findings of PFOS induced hypothyroxinemia (including in pregnant animals) should really be discounted. Furthermore, it is not clear if other PFAs cause the same negative bias as PFOS in analog T4 assays. Given the current state of knowledge, where PFAs have been shown to perturb thyroid metabolism genes, [28] displace T4 from its carrier proteins, [29, 30, 43] and to affect total T4 concentrations even when free T4 is deemed to be unaffected, [27] the current study makes a valuable contribution to the

controversy in ruling out the possible hypothyroxinemic inducing effect of PFAs during pregnancy.

## 3. 5. Conclusion

The data from this study suggests that perfluorinated acid concentrations are not determinants of maternal hypothyroxinemia at background exposure concentrations typical of this population in the Canadian city of Edmonton and its surroundings. Concentrations of PFAs have been steadily declining since 3M's phase out of PFOS in 2002, and subsequent legislation from the Canadian (PFOS labeled as 'toxic' under the Canadian Environmental Protection Act) and American governments (two Significant New Use Rules with PFOS and other PFAs) have resulted in decrease PFA usage. There are currently no federal safety standards for PFOA in the US [55] and Health Canada and Environment Canada are currently performing screening assessment for PFOA.[56] However, as of May 2009, PFOS was included in Annex B of the Stockholm Convention on persistent organic pollutants. [57] Thus, given that the risk of hypothyroxinemia with PFA exposure appears to be negligible today in the studied population, it will likely continue to remain so as the concentrations of PFAs in the environment decline.

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# CHAPTER 4. **Public Health Relevance**

From our study, the relationships between hypothyroxinemia and PFA exposures do not show significant risks, but PFHxS' involvement could be considered unclear at higher concentrations, and this study is the first of its kind. Furthermore, subpopulations in Alberta have been identified to have PFHxS serum concentration in the 100s of ng/mL, much higher than studied here. [24] Aside from hypothyroxinemia, PFAs have been found to be involved with other effects, such as low birth weight in humans and in animal models. Despite the lack of evidence from past human research, potential effects should be recognized from animal studies. More human studies based on biologically plausible endpoints, supported by information from animal research, may help regulate emissions to the ambient environment and provide greater safety standards to occupational workers. Additionally, these chemicals are virtually ubiquitous in all people. Prevention of risks to children and future generations start from decreasing risk to the pregnant mother and thus the fetus since these chemicals have been shown to cross the placental barrier.

## 4. 1. Study Limitations

### 4. 1. 1. Chan et al.Study

#### 4. 1. 1. 1 Confounding Factors

Although the data in this report do not appear to support PFA's involvement with maternal hypothyroxinemia outcome in humans, results should be interpreted carefully. Because we do not truly understand the extent of PFA influence on human thyroid hormone and disease status, a number of things such as confounding factors and physiological events may impede observing the possible relationship. This was observed in several studies, such as with TPO-Ab's relationship with lower free T4,[17] and neuropsychological outcome. We did not measure for TPO-Ab. Future studies involving hypothyroxinemia and PFA exposure may benefit from taking particular interest in this antibody and its effects on disease outcome.

Further study limitations include the small number of participants in our study (n=273). The Emmett et al. study, [19] which examined thyroid hormone disease and PFA exposure, utilized 371 subjects in their research, while other studies examining outcomes such as low birth weight from PFA exposure have had subjects numbering 1440.[12] We may have benefited from a greater number of study subjects to truly achieve the greatest statistical power.

#### 4. 1. 1. 2 Iodine Availability

We did not measure for iodine concentrations in our study. Other studies such as the Pop et al. research, purposely stated that maternal samples were collected from iodine sufficient areas, in examining hypothyroxinemia and

neurodevelopmental outcome in children.[17] Pop et al.may have chose to do this as the association between hypothyroidism induced by iodine deficiency during pregnancy, and mental retardation in the offspring have been recognized for over 100 years.[20] Further, iodine deficiencies in the mother and fetus have complicated the question of whether the fetus' mental retardation was influenced by maternal or fetal hypothyroidism.[21] Iodine measurements would rule out potential confounding factors, such as hypothyroxinemia or hypothyroidism induced conditions from iodine insufficiency. However, it could also be assumed that these measurements would not be necessary in studies in developed countries, as iodine (from products such as iodinized salts) would be assumed to be readily available. [21]

## 4. 2. Avoiding Analytical Systematic Error

We further reported on the usage of proper mass transition ratios for PFHxS.[22] This is vital as future research could possibly give more insight into how much of an impact the contaminant could have on possible disease outcome. Past research has mostly relied on using the *m/z* 399/80 and *m/z* 399/99 transitions, and it is difficult to gauge how much of an influence these reported concentrations have on physiological functions in humans and animals. By using the interference free transition to report PFHxS concentrations, we can have a more accurate idea of the acid's effects. This is especially important in our sample set, because steroid sulfate interferences were found to be highest in pregnant women.[22]

## 4. 3. Regulations

Canada was the first country to take precautionary action on certain perfluorinated acid precursors by banning four fluorinated polymers which contained telomer alcohols which are precursors to perfluorinated carboxylic acids such as PFOA, in the environment. On February 2006, Environment Canada and Health Canada proposed a Risk Management Strategy for the fluorotelomers, with recommendations for permanent bans. These recommendations were acted upon, and regulations on banning the manufacture, sale and importation of these chemicals were also proposed. Critics however, argue that the regulations do not apply to imported products containing these chemicals, which could potentially leave Canadians exposed. As of recent, both departments have begun talks on Proposed Action Plan for Assessment and Management of Perfluorinated Carboxylic Acids and Precursors. [23]

In 2004, Health Canada and Environment Canada recommended that PFOS and its salts and precursors be labeled 'toxic' by the Canadian Environmental Protection Act (CEPA) definition, meaning the chemical may have an immediate or long term harmful effect or constitute danger to the environment. PFOS was added to the 'List of Toxic Substances' under CEPA, and intentions for regulation were put in place during July 2006. More specifically, these regulations would

prohibit manufacturing, sales and importations of PFOS and PFOS containing products.[23]

Internationally, U.S. regulators have reached a voluntary agreement with eight companies to phase-out the use of PFOA, after the reports of an expert advisory panel to the U.S. Environmental Protection Agency found the chemical to be a likely carcinogen. Here, companies will reduce PFOA emissions by 95% in 2010 and try to eliminate sources by no later than 2015.[23]

The major U.S. manufacturer of PFOS, 3M, voluntarily stopped manufacturing PFOS related chemicals by 2003, and since 2000 the U.S. EPA has imposed a PFOS ban except for in specialized uses for aviation, photography and microelectronics. Similarly, Sweden proposed a global ban on PFOS under the Stockholm Convention on Persistent Organic Pollutants and now PFOS is included in Annex B. However, chemicals listed under Annex B are not banned outright, and production of PFOS is still allowed for many permitted applications. Despite these restrictions however, China is currently continuing and expanding its manufacture of PFOS. The European Commission (EC) sent a proposal for a Directive for PFOS restriction in carpets, textiles and clothing but the results of this are expected to take a few years.[23]

## 4. 4. Conclusion

This research adds to the early body of literature on PFA analysis and human health effects. It is the only study to have examined for effects on maternal hypothyroxinemia resulting from exposure to any PFA, and this is among the first studies to examine for any human health effects of PFHxS. Although we did not find a significant relationship between PFA exposure and hypothyroxinemia, studies in higher exposed subpopulations may be warranted in future research.

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