Developmental Toxicity from Perfluoroalkyl Acid and Mercury Co-Exposure in Experimental and Epidemiological Models

by

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Abstract

Perfluoroalkyl acids (PFAAs) are synthetic contaminants that are widespread in the environment and routinely detected in human blood. Their prevalence raises concerns over their potential adverse implications on human health, especially from exposure during sensitive stages of development (e.g., pregnancy). PFAAs also share common sources of exposure with mercury (Hg) but the combined effects from co-exposure to these contaminants have yet to be elucidated. Here, it was hypothesized that prenatal exposure to PFAAs would result in deficits in offspring neurological development. These effects were demonstrated in offspring using an experimental model of directly dosed pregnant rats and associated effects in humans were examined in children of participants from a prospective birth cohort. It was further hypothesized that associations and effects from prenatal PFAA exposure would be modified when considering the interaction from co-exposure of PFAAs with Hg, and these associated changes in child neurodevelopment are linked to altered maternal thyroid hormones during pregnancy.

Pregnant rats exposed perfluorooctane sulfonate (PFOS) and/or methylmercury (MeHg) were used to determine effects on offspring neurodevelopment. Co-exposure to both PFOS and MeHg induced additive effects in newborns that contrasted with combined exposure results in juveniles. In older offspring, individual exposure to PFOS- or MeHg-alone induced significant changes in behaviour that were not evident in control or combined exposure groups, an antagonistic response mirrored by alterations in measured brain metabolites. This is the first investigation to use a target metabolomics platform to explain changes in offspring behaviour from combined maternal exposure to organic contaminants and heavy metals, providing evidence of a chemical interaction between PFOS and MeHg.

A longitudinal study design was used to investigate time-dependent associations of PFAAs with thyroid hormones over each trimester of pregnancy and postpartum, considering additional stress of Hg co-exposure in the APrON birth cohort. Perfluorohexane sulfonate (PFHxS) and branched isomers of PFOS were positively associated with TSH, with the strongest associations early in gestation. Higher perfluoroalkyl sulfonate exposures associated with higher TSH and/or lower FT4 are strongly suggested to be risk factors for subclinical maternal hypothyroidism. This was one of the first investigations to observe PFOS isomer-specific associations with maternal thyroid hormones, an important consideration for future studies as these associations may be overlooked when only considering crude measures of 'total-PFOS'.

To evaluate associations with child neurodevelopment, PFAAs collected during the second trimester of pregnancy and interaction with Hg were modeled with child development outcomes at 2-years-old. PFAAs were negatively associated with cognitive and social-emotional development scores. Specific PFOS isomers were associated with child language scores, associations that were determined to be non-linear. Other modeled PFAAs were revealed to be associated with child motor and social-emotional scores only when considering Hg-interaction, suggesting that significance of PFAA-neurodevelopment associations are potentiated by the presence of Hg.

This thesis provides evidence of neurodevelopmental toxicity of PFAAs in experimental and epidemiological models. In animals, combined exposure to PFOS and/or MeHg alters the behaviour and brain chemistry of offspring. In humans, prenatal exposure to PFAAs and corresponding isomers disrupt maternal thyroid hormones during pregnancy, exacerbated in women exhibiting thyroid hormone dysregulation, or modified by Hg co-exposure that may explain PFAA-associated changes in child neurodevelopment.

Preface

Chapter 2 was part of an internal collaboration with Dr. Karim Fouad of the Department of Physical Therapy (University of Alberta) and international collaboration with Dr. Jonathan Benskin of the Department of Environmental Science and Analytical Chemistry (University of Stockholm, Sweden). All protocols and procedures in Chapter 2 were approved by the animal care and use committee at the University of Alberta, project name "Rodent neurodevelopment and contaminants", AUP00000809, approved 22 Sept 2013, and renewed annually. I was responsible for experimental design, ethics approval, and project oversight (animal testing, tissue and data collection, chemical analysis (perfluroooctane sulfonate), and all written work). Jacqueline Karathra assisted with animal husbandry and animal behaviour testing. Jonathan Benskin and Anton Ribbenstedt mentored and assisted with metabolomics data collection and analysis. Drs. Amy MacDonald and David Kinniburgh of the Alberta Centre for Toxicology (University of Calgary) conducted mercury analysis. Dr. Trevor Hamilton provided video and animal tracking software for behaviour testing. Dr. Karim Fouad provided guidance on animal behaviour testing and tissue collection, including providing the facilities and necessary scientific apparatus. Jonathan Martin, my supervisor, assisted with the overall experimental design.

Chapters 3 and 4 were conducted as ancillary investigations within the Alberta Pregnancy Outcomes and Nutrition (APrON) cohort. All protocols within the APrON study were approved by the Health Research Ethics Board from the University of Calgary (Ethics ID: REB14-1702_REN3) and the Human Ethics Research Board from the University of Alberta (Study ID: Pro00002954). Jonathan Martin, my supervisor, was actively involved in experimental design and writing. The APrON cohort involved thousands of women from the metropolitan areas of Calgary and Edmonton; participant recruitment, data and sample collection and integration were led by Dr. Deborah Dewey and APrON team members.

Within Chapter 3, I was responsible for method development and subsequent analysis of PFAAs in maternal plasma of APrON participants, as well as data consolidation and written work. Drs. Elham Khodayari-Moez and Irina Dinu of the School of Public Health (University of Alberta) conducted statistical analysis. Susan Goruk and Dr. Catherine Field of the Department of Agricultural, Food and Nutritional Science (University of Alberta) conducted thyroid hormone

analysis and Drs. Amy MacDonald and David Kinniburgh of the Alberta Centre for Toxicology (University of Calgary) conducted mercury analysis.

Within Chapter 4, I was responsible for experimental design, analysis of PFAAs in maternal plasma from APrON participants. Data collection and consolidation from was led by Dr. Deborah Dewey and members of the APrON team. Morteza Hajhosseini and Dr. Irina Dinu of the School of Public Health (University of Alberta) conducted statistical analysis. Drs. Amy MacDonald, and Dr. David Kinniburgh of the Alberta Centre for Toxicology (University of Calgary) conducted mercury analysis.

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List of abbreviations

(F)T3	(Free) Triiodothyronine
(F)T4	(Free) Thyroxine
AA	Amino acid
AC	Acylcarnitine
ACN	Acetonitrile
ADHD	Attention deficit hyperactivity disorder
AM	Arithmetic mean
ANOVA	Analysis of variance
APrON	Alberta Pregnancy Outcomes and Nutrition (Canadian cohort)
BA	Biogenic amine
Bayley-III	Bayley scale of infant and toddler development (3rd edition)
BMI	Body mass index
CAD	Canadian dollar
CER	Ceramide
CH_3HgCl	Methylmercury chloride
CHMS	Canadian Health Measures Survey (Canadian cohort)
CI	Confidence interval
DMSO	Dimethyl sulfoxide
DNBC	Danish National Birth Cohort
ECF	Simons electrochemical fluorination
EFSA	European Food Safety Authority
GC-MS/MS	gas chromatography-tandem mass spectrometry
GD	Gestational day
GM	Geometric mean
Hg	Mercury
HPLC-MS/MS	High performance liquid chromatography-tandem mass spectrometry
HPT	Hypothalamic-pituitary-thyroid
ICP-MS	Inductively coupled plasma mass spectrometry
INMA	INfancia y Medio Ambiente (Spanish cohort)

IQ	Intelligence quotient
IU	International units
KEGG	Kyoto Encyclopedia of Genes and Genomes (database)
LOAEL	Lowest observed adverse effect level
LOD	Limit of detection
LOQ	Limit of quantitation
LysoPC	Lysophosphotidylcholine
MDL	Method detection limit
MeHg	Methylmercury
MeOH	methanol
mg/kg bw/d	Milligram per kilogram of body weight per day
MIREC	Maternal-Infant Research on Environmental Chemicals (Canadian cohort)
MQL	Method quantitation limit
NHANES	National Health and Nutrition Examination Survey (US cohort)
NMDA	N-methyl-D-aspartate
OC	Organochlorine
PBDE	Polybrominated diphenyl ethers
PC	phosphotidylcholine
PCA	Principal component analysis
PCB	Polychlorinated biphenyls
PERFOOD	PERfluorinated organics in our diet (European Union cohort)
PFAA	Perfluoroalkyl acid
PFAS	Perfluoroalkyl substances
PFCA	Perfluoroalkyl carboxylic acid
PFSA	Perfluoroalkyl sulfonic acid
PLS-DA	Partial least-squares discriminant analysis
PND	Postnatal day
POP	Persistent organic pollutant
PPAR	Peroxisome proliferated activated receptors
ppb	Parts per billion

ppm	Parts per million
QA/QC	Quality assurance and quality control
RBC	Red blood cell
RCF	Relative centrifugal force
RPM	Revolutions per minute
RSD	Relative standard deviation
SD	Standard deviation
SE	Standard error
SM	Sphingomyelin
TBG	Thyroid binding globulin
TDI	Tolerable daily intake
THg	Total mercury
TPOAb	Thyroid peroxidase antibodies
TRH	Thyrotropin-releasing hormone
TSH	Thyroid stimulating hormone
TTR	Transthyretin
UHPLC-MS/MS	Ultra-high-performance liquid chromatography-tandem mass spectrometry
US EPA	United States Environmental Protection Agency
Abbreviated linear and branched perfluoroalkyl acids	
PFBA	Perfluorobutanoate
PFBS	Perfluorobutane sulfonate
PFPA	Perfluoropentanoate
PFHxA	Perfluorohexanoate
PFHxS	Perfluorohexane sulfonate
PFHpA	Perfluoroheptanoate
PFOA	Perfluorooctanoate
PFOS	Perfluorooctane sulfonate
PFNA	Perfluorononanoate

PFDA	Perfluorodecanoate
PFDS	Perfluorodecane sulfonate
PFUnA	Perfluoroundecanoate
PFDoA	Perfluorododecanoate
PFTrA	Perfluorotridecanoate
PFTeA	Perfluorotetradecanoate
L-PFOA	Linear perfluorooctanoate
∑Br-PFOA	Total branched-perfluorooctanoate isomers
6*m*(*iso*)-PFOA	*Iso*-perfluorooctanoate
5*m*-PFOA	5*m*-perfluorooctanoate
4*m*-PFOA	4*m*-perfluorooctanoate
3*m*-PFOA	3*m*-perfluorooctanoate
∑*dm*-PFOA	∑*Dimethyl*-perfluorooctanoate
L-PFOS	Linear perfluorooctane sulfonate
∑Br-PFOS	Total branched-perfluorooctane sulfonate isomers
6*m*(*iso*)-PFOS	*Iso*-perfluorooctane sulfonate
5*m*-PFOS	5*m*-perfluorooctane sulfonate
∑3*m*+4*m*-PFOS	∑3*m*+4*m*-perfluorooctane sulfonate
1m-PFOS	1*m*-perfluorooctane sulfonate
∑*dm*-PFOS	∑*Dimethyl*-perfluorooctane sulfonate

Chapter 1

Introduction

1.1 Exposure to Environmental Contaminant Mixtures

Humans are exposed to a wide array of chemical contaminants that are present in the environment. Chemical exposure occurs through various means, whether inhaled from the air, ingested in food and water, and through dermal contact with commonly used commercial products. Regulatory agencies commonly consider the resultant effects from exposure to single chemicals in human risk assessment, as exposure to complex contaminant mixtures present a major challenge that is rarely addressed[1]. Biomonitoring studies revealed that exposure is not constant in rate, nor magnitude of occurrence, and specific populations may be exposed to higher levels of individual contaminants, with intermittent periods of higher exposure throughout a given lifetime[2]. For example, Cree First Nations women from northern Quebec have higher blood levels of mercury compared to average Canadians due to increased dietary intake of locally sourced fish[3]. Although not all exposures will be harmful, screening for mixtures is necessary to identify chemicals and combinations thereof that may pose risk to human health, especially during sensitive stages of growth and development (e.g., pregnancy). Investigating contaminant mixtures in both controlled toxicological investigations (animal models), and through observations studies of environmentally relevant concentrations (human biomonitoring) will give insight into the relative contributions of these contaminants to adverse human health and development of disease.

In the scientific community, there has been recent interest in identifying effects from exposure to environmental contaminants during pregnancy. In Canada, a recent cohort, the maternal-infant-research on environmental chemicals (MIREC), reliably detected 28 individual contaminants that fell into broader classes of metals, plasticisers, perfluoroalkyl substances (PFASs), polychlorinated biphenyls (PCBs), and organophosphate and organochlorine (OC) pesticides in women from across Canada[4]. Heavy metals, such as lead and mercury are well-studied neurotoxicants, known to have adverse effects on brain function and/or brain development. There is considerably less data on the effects from exposure to persistent organic pollutants (POPs) such as PFASs and organochlorines. A multi-pollutant assessment of both

PFASs and OCs has demonstrated the potential for endocrine disruption, as both compound classes disrupted maternal thyroid hormones during pregnancy[5]. Among the broader class of analyzed POPs, PFASs are among the most persistent, and present in the highest levels in the blood of Canadians[4].

1.2 PFAA Sources and Environmental Distribution

1.2.1 Applications, Use, and History of Manufacture

Perfluoroalkyl acids (PFAAs) are a subgroup of perfluoroalkyl substances (PFASs) containing a fully fluorinated (perfluoroalkyl) carbon chain and an acidic functional group, such as a carboxylic or sulfonic acid moiety. Being relatively strong acids, PFAAs exist in most environments as the negatively charged deprotonated conjugate base (i.e., carboxylate or sulfonate). These man-made organic chemicals have been used in numerous commercial and industrial applications, including as stain repellent surface coating for clothing, textiles, and food packaging, or as surfactants in paints, lubricants, fire-fighting foams, or in the production of fluorinated polymers[6,7].

The physicochemical properties and manufacturing history of PFAAs have been the subject of extensive review[8–10]. In brief, PFAAs have high chemical and thermal stability, and they have both hydrophobic and lipophobic properties, making them unique surfactants that are particularly useful in extreme conditions where hydrocarbon surfactants would degrade, such as under high heat or extreme pH[11]. Large-scale manufacturing of perfluorinated chemicals was initiated in the 1950s by the 3M Company, primarily through a patented process called Simons Electrochemical Fluorination (ECF)[12]. The ECF process begins with a hydrocarbon feedstock, and fragmenting of the carbon backbone, resulting in PFAS products with a range of chain lengths and mixtures of linear and branched isomers (typically ~70 % linear and 30 % branched)[13–15]. Perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) were the most commonly employed for consumer and industrial purposes. Long-chain PFAAs are the most surface active, and for purposes of this discussion will be defined as perfluoroalkyl carboxylates with at least 7 perfluoroalkyl carbons (PFCAs, $C_nF_{2n+1}COOH$ with n > 7) and perfluoroalkyl sulfonates with at least 6 perfluoroalkyl carbons (PFSAs, $C_nF_{2n+1}SO_3H$ with n > 6)[16]. The

names, formulae, and abbreviations of PFAAs investigated in this thesis are listed in Table 1-1, and the structures of PFCAs and PFSAs are depicted in Figures 1-1A and 1-1B, respectively.

1.2.2 Environmental Prevalence

The unique persistent properties of PFOS and PFOA allow for their long-range transport, dispersing from their emission sources throughout the environment. PFAA emissions may be considered **direct** from intentional manufacturing sources and use of associated products, or **indirect**, such as from residual impurities or degradation of labile precursor PFASs[8,9]. PFAAs have even been detected in remote regions of the Arctic, and are hypothesized to have been transported long distances through ocean currents, as well as through the atmosphere or as semi-volatile PFAS precursors[17–22]. Moreover, PFAAs are detectable today in all environmental matrices, including air, water, sediments of lakes, rivers and seas, and in wastewater biosolids that are applied in agriculture. In fact, edible crops can uptake PFAAs from biosolid-amended soils[23,24], representing one of many environmental pathways of indirect human exposure. PFAAs also bioaccumulate in fish, becoming more concentrated when progressing up the aquatic food chain, providing a major source of dietary exposure for people and other top predators in the marine environment.

1.2.3 Early Detection and Quantification of PFAAs

Pioneering scientific investigations alerted the world to the presence of organic fluorine in human serum as early as 1968[25], and by 1974 these unknown fluorinated compounds were reported in both maternal and umbilical cord blood, indicating their capacity to cross the placenta and enter fetal circulation[26]. This organic fluorine was also detected in the serum of fluorochemical workers[27] and the general population[28] in the 1980s. More than thirty years after the initial report, Hansen et al. from the 3M Company, confirmed and quantified several PFAAs in human serum by using high performance liquid chromatography-tandem electrospray mass spectrometry (LC-MS/MS)[29]. Soon thereafter, Giesy and Kannan, funded by the 3M Company, reported the global presence of PFOS and other fluorinated compounds in samples of liver, eggs, muscle or blood plasma of various wildlife species[30].

1.3 Human PFAA Biomonitoring

1.3.1 Biomonitoring and Regulation, Production Phase-out

Biomonitoring of occupationally exposed fluorochemical workers from 3M manufacturing sites in Antwerp Belgium[31], and in Decatur, USA[32] in 2003 by Olsen et al. revealed high serum levels of PFOS, PFOA, and PFHxS, (e.g., mean serum PFOS in males ranged from 0.96 -1.40 parts per million (ppm)[31]). Around the same time, serum PFAA concentrations in the general US population were also reported to be several orders of magnitude lower than occupationally exposed workers (e.g., median serum PFOS = 35.8 parts per billion, (ppb))[32]. Longitudinal monitoring of retired fluorochemical workers also revealed the long serum elimination half-lives of PFHxS, PFOS, and PFOA in humans (8.5, 5.4, and 3.8 years, respectively)[33]. Biomonitoring programs for PFAAs are now established in many countries, including Canada (Canadian Health Measures Survey, CMHS)[34], the US (National Health and Nutrition Examination Survey, NHANES)[35] and the European Union (PERfluorinated organics in our diet, PERFOOD)[36].

In 2002, the major historic manufacturer of PFAAs (3M Company) began a voluntary phase-out of PFOS, PFOS-precursors, and PFOA due to emerging concerns over the environmental distribution and possible health risk of long-chain PFAAs[37]. Although production of PFOA was scaled up by other companies at that time, a stewardship program launched by the US EPA in 2006[38,39] led to the agreed phase-out and elimination of PFOA and longer chain PFOA precursor compounds by 2015.

Although production of these long-chain PFAAs has been largely discontinued in North America and Western Europe, trends of production and emissions are increasing in China[40,41] and India[9]. In addition to the voluntary production phase-out, PFOS and its precursors were added to Annex B of the *Stockholm Convention on Persistent Organic Pollutants* in 2009[42], and PFOA and PFHxS are currently under review. Despite the phase-out and restrictions from regulatory agencies, PFAA production continues and is even increasing in developing countries. The listing of PFOS and its precursors under Annex B provided many exemptions for countries to continue producing these problematic substances. The global emission profiles and extent of

distribution from these sources have yet to be fully elucidated, representing an ongoing source of exposure for humans and the environment.

As a general trend, PFAS manufacturers have been replacing long-chain PFAAs with shorter-chain analogs in consumer and industrial applications. For example, when 3M Company phased out PFOS and its precursors (i.e., C_8), it immediately replaced this chemistry with perfluorobutyl substances (i.e., C_4). This practice has been encouraged by the lower bioaccumulation potential of shorter chain length PFASs[43], which might decrease their impact on the environment or human health compared to longer chain PFAAs[44,45]. However, concerns have been raised over their use because they have not been well studied[46,47], and these replacement compounds are still environmentally persistent[48,49].

1.3.2 Human Exposure Pathways

Humans are exposed to PFAAs from numerous sources, including dietary intake[50–55], drinking water[55], and from the presence of PFAAs and their precursor compounds in carpeting, textiles, indoor air and household dust[56–60]. There is a large body of evidence to support dietary intake as the primary route of human PFAA exposure (see reviews by Domingo et al. and Pico et al.[61–63]). Recent investigations of PFAA levels in various dietary items in Europe found that fish and seafood have the highest concentrations[64,65]. Populations with higher rates of fish consumption have higher serum levels of PFAAs[66–68].

There are also scenarios of high human exposure from treated carpeting or household dust. For example, a Canadian family was found to have disproportionately high levels of PFHxS (range 27.5 – 423 ng/mL), PFOS (range 15.2 – 108 ng/mL) and PFOA (range 2.4 – 9.23 ng/mL), and it was established that their household carpeting had been routinely treated with aftermarket surface-treatment applications of Scotchgard™ over a 15-year period[69].

In 2008, the European Food Safety Authority (EFSA) Scientific Panel on Contaminants established recommended values for human tolerable daily intake (TDI) based on the culmination of available data for PFOS (TDI=150 ng/kg bw/d) and PFOA (TDI=1500 ng/kg bw/d)[70], the two major and most frequently detected PFAAs. A follow-up report in 2012 determined average daily exposure levels of 5.2 and 4.3 ng/kg bw/d in adults, and 10 and 7.7 ng/kg bw/d in toddlers for PFOS and PFOA, respectively[71]. Although these values are well below the

recommended TDI, the guidelines do not mention intake for specific sensitive subpopulations, such as for pregnant women and the developing fetus.

1.3.3 Temporal Trends of Human Exposure

Changes in manufacturing practices beginning in the early 2000s correspond with subsequent trends of PFAA concentrations in human serum, notably the relatively rapid decline of PFOS[72–77]. For example, cross-sectional analysis of blood plasma from American Red Cross donors showed that PFOS declined from 34.9 ng/mL in 2000 to 8.30 ng/mL by 2010[74]. However, trends in PFHxS in the US were less consistent, initially declining between 1999 and 2006, but then increasing until 2008[75]. Increasing trends of PFHxS during the same time period were also observed in pregnant women from Uppsala, Sweden[78]. China identified PFHxS as the second-most abundant PFAA, after PFOS, in both general[79] and occupationally exposed[80] populations. Non-linear trends were also observed for PFCAs, as serum PFOA initially decreased in the US population between 1999 and 2003[73], but then was unchanged between 2003 and 2008. A concerning trend is that perfluorononanoic acid (PFNA) continually increased over this entire time period[75]. Biomonitoring of PFHxS, PFOS, PFOA and PFNA in the Canadian Health Measures Survey (CHMS) in 2011 showed similar concentrations to previous values recorded in the US in 2008[81]. PFAA Exposure in the Canadian population was examined again in 2016/2017 (CHMS fifth cycle), and this record is expected to be published in the near future[82].

1.4 Toxicology of PFAAs

Experimental models have been used extensively to determine the toxicological effects of PFAAs, an essential step in establishing regulations regarding human health and safety. Such models provide useful information on toxicokinetics and developmental toxicity, including neurobehavioural and neurochemical changes in offspring exposed in utero. Past studies of PFAAs have primarily focused PFOS and PFOA, and will be used as primary examples, but data from other PFAAs will also be discussed based on availability.

1.4.1 Absorption, Distribution, Metabolism and Excretion of PFAAs

Dietary intake is generally considered the primary route of human PFAA exposure. PFAAs are well absorbed in the gastrointestinal tract, as mice were found to absorb 98.8 % of

radiolabeled [^{14}C]PFOA after 48 hours[83]. Similar results were obtained with PFOS, with 99 % of [^{14}C]PFOS recovered in carcass, urine, plasma and red blood cells of rats 24 hours after exposure[84]. Even at high doses (e.g., 4.2 mg/kg of PFOS) uptake was extremely efficient[84].

Although PFAAs tend to accumulate in the serum, liver, and kidney, they will distribute to a lesser extent to adipose tissue, lungs, heart, and brain[85]. Investigations in large domestic mammals (sheep, cows and pigs) given PFAA-contaminated feed (e.g., PFBS, PFHxS, PFHpS, PFOS, PFHxA, PFHpA, and PFOA) observed an accumulation of PFAAs in the serum fraction of whole blood, with PFOS also accumulating in the liver[86–88]. The distribution of PFAAs to serum is thought to be related to non-covalent binding to serum proteins, including albumin[89–91] speculated to competitively displace endogenous ligands, such as fatty acids and steroid hormones.

After absorption and distribution, PFAAs are not known to be metabolized, contributing to their slow rates of clearance in humans and in animal models. Humans have the slowest PFAA elimination rates of any mammalian species studied. For example, the calculated half-life of PFOS in occupationally exposed humans was 5.4 years[33], compared to 131-200 days in monkeys[84,92], and 38-62 days in rats[84]. Humans have faster excretion of short-chain PFAAs (e.g., PFBA = 26 days)[93] compared to specific long-chain PFAAs (PFHxS, PFOS, and PFOA > 5 years)[33]. The half-life corresponds, in part, to the route of PFAA elimination, as for most organic acids renal clearance (kidney) is a major driver of observed toxicokinetics (see Han et al. for review[94]). For most mammals, short-chain PFAAs (C_4 to C_6) exhibit increased renal clearance when compared to longer chain PFAAs (C_8 and C_9)[94], which are speculated to be eliminated with assistance from renal transport proteins[94]. However, in humans, PFOA and PFOS are poorly eliminated in urine, and preferentially eliminated in feces through biliary excretion[95]. Treatment with cholestyramine (a bile acid sequestrant) significantly increased the rate of fecal elimination of PFHxS, PFOS, and PFOA, providing evidence of their biliary excretion[96] but also demonstrating that fecal elimination is slow because of reuptake in the intestine. Such enterohepatic recirculation and slow renal clearance result in the long half-lives for PFHxS, PFOS and PFOA in humans.

1.4.2 Fetal and Newborn PFAA Exposure

An early study with rats investigating maternal transfer of PFOA found that high repeated doses to dams (3-30 mg/kg bw/d) starting on gestational day (GD) 4 resulted in detectable levels of PFOA in the placenta, amniotic fluid, embryo and milk[97]. Further studies of repeat exposure during gestation with mice confirmed substantial in utero transfer of PFAAs. For PFOA, newborn pups had a higher detectable serum concentration when compared to their corresponding dams[98,99]. For PFOS, newborn pups were found to have comparable serum levels to maternal dams[84,100]. During the postnatal period, PFAAs continue to be transferred to newborns by dams from lactation, as indicated by rat cross-fostering studies that found detectable levels of PFOS in pups from untreated control litters that were nursed by PFOS-exposed dams[101].

In humans, numerous studies have demonstrated the potential for transplacental transfer of PFAAs through biomonitoring of paired umbilical and maternal serum samples[102–113]. However, different PFAAs cross the placenta to different degrees. For example, a U-shaped trend for transfer efficiency was observed for a series of PFCAs[109]. Transfer efficiency (the ratio of PFAA concentration in cord blood to that in maternal blood) decreased with increasing perfluoroalkyl length (1.2 – 0.25, for C_7 to C_{10}), but increased again with longer chain lengths (0.25 – 1.76 from C_{10} to C_{13})[109]; a similar trend was also evident for a series of PFSAs[111].

Furthermore, detailed investigations including isomers of PFOS and PFOA (listed in Table 1-2, depicted in Figure 1-2, and chromatograms present in Appendix B) found that umbilical cord blood was relatively enriched in branched isomers[108,114,115], a finding that was further confirmed in vitro[116]. These patterns are speculated to be influenced by the relative binding affinities to serum proteins, such that those PFAAs with the highest affinity for serum proteins do not cross as easily as those PFAAs that are bound more weakly[114].

Branched PFAA isomers (e.g., PFOS, PFOA, and PFNA) are also detectable in wildlife and in environmental samples[117–121]. However, their toxicity relative to the predominant linear isomer has not been thoroughly investigated. This is a knowledge gap because human populations have a wide range of isomer proportions. For example, the branched PFOS isomer content (% branched) has been reported in China (52 %)[122], Norway (30-50 %)[72,123], Vietnam (19 %)[124], and Canada (31 %) (see Chapter 3 results). Despite this knowledge, and the finding that

branched isomers cross the placenta to a greater extent than linear isomers, there is a paucity of isomer-specific PFAA data in epidemiological studies.

1.4.3 Experimental Models of Developmental Toxicity

Lau et al. reviewed the developmental toxicity of PFAAs in the early 2000s[125,126], reviews that were more recently expanded on by Stahl et al.[127] and Abbott[128]. It was reported that repeat daily doses of PFOS in rabbits throughout gestation resulted in decreased maternal (1 and 2.5 mg/kg bw/d) and fetal (2.5 and 3.75 mg/kg bw/d) weight[129]. A two-part investigation by Thibodeaux et al. of the prenatal and postnatal effects reported decreased maternal weight gain and reduced feed consumption at higher doses of PFOS (2 to 10 mg/kg bw/d), and offspring from the highest dose group (10 mg/kg bw/d) also had significant weight reductions that were accompanied by increased incidence of cleft palate and cardiac defects[130]. In the second part of the investigation, live-born rats were found to have a dose-dependent reduction in postnatal survival, as pups shortly after birth became pale, inactive, and moribund, with mortality rates of 100 %, 95 %, 60 % and 40 % at 10, 5, 3, and 2 mg/kg bw/d dose groups, respectively; The survival rate of the 1 mg/kg bw/d dose group was not significantly different from controls[100]. Decreased weight gain in both dams and pups, and dose-dependent increases in pup mortality were supported in follow-up investigations with decreased daily doses of PFOS, as 100 % pup mortality in the highest 3.2 mg/kg bw/d dose group was reduced to 0 % when lowering the dosing level to 1.2 mg/kg/d[101,131]. The details provided in these investigations were used to establish appropriate dosing ranges for PFOS exposure during gestation and lactation in my own developmental toxicity investigation (see Chapter 2).

1.4.4 Developmental Neurotoxicity

In animal models, PFAAs preferentially accumulate in liver and blood but can also be detected, albeit to a lesser extent, in brain tissue. Distribution into the brain may be limited by the protective effect of the blood brain barrier[132]. Although data are limited on PFAA distribution to the human brain, one study in Italy did demonstrate PFAAs crossing the blood brain barrier, but there were lower concentrations in brain tissue (0.5 and 1.3 ng/g) compared to whole blood (3.0 and 5.1 ng/g for PFOA and PFOS, respectively)[133]. However, during pregnancy, PFAAs are known to cross the placenta and may subsequently be transferred more efficiently through an

immature fetal blood brain barrier. For example, in rats examined toward the end of gestation (GD 20), neonatal rats had higher brain to plasma ratios of PFOS (0.41) compared to maternal rats (0.04) dosed at 1 mg/kg bw/d[134]. Thus, newborn rats may have an increased risk of adverse neurodevelopment from increased in utero exposure due to increased permeability of the fetal blood brain barrier.

Prenatal exposure to PFOS induced early neurodevelopment delays in newborn rats and mice, including delayed eye opening and pinna detachment[100,131], as well as delays in surface righting reflex[135]. In older juveniles, PFOS exposed male rats had increased locomotor activity and decreased habituation behaviour when placed in a novel testing environment (open field arena) compared to untreated controls[136]. However, in utero PFOS exposure was not observed to affect subsequent cognitive function, with no observable effects on learning and memory (e.g., auditory startle response, swim maze or T-maze)[100,136]. Prenatal exposure to PFHxS was also not observed to have any effect on the development of offspring, even at high doses up to 10 mg/kg bw/d[137].

Recent studies have attempted to identify molecular targets to explain PFAA-mediated changes in neurodevelopment. Increased motor activity, referred to as hyperactivity, was induced in control rat pups administered a nicotine injection, whereas nicotine did not induce any effects in pups exposed to a single postnatal dose of PFOS, PFOA[138], or PFHxS[139]. The lack of nicotine-induced hyperactivity in postnatal PFAA-exposed rat pups suggested that PFAA-induced effects are associated with changes in cholinergic neurotransmission[138–140]. Chronic perinatal exposure to PFOS was found to affect expression of calcium-related signal molecules[141,142], and alter the expression of proteins involved in synaptic transmission[143]. Single postnatal PFOS exposure in offspring during the brain growth spurt, a critical period of rat pup brain development, affected transcription of specific genes involved in dopaminergic transmission[144].

Single high doses and low chronic doses of PFAAs to pregnant dams have been observed to affect neurological development of offspring. In Chapter 2, I use a battery of behavioural tests to examine the extent of PFOS-induced neurotoxicity from chronic perinatal exposure, as well as a target metabolomics platform to determine if PFOS-specific changes in behaviour were associated with altered metabolite profiles (lipids, amino acids, and biogenic amines) in brain regions.

1.5 Methylmercury (MeHg) Co-exposure with PFAAs

1.5.1 MeHg Sources, Distribution, and Human Exposure

Many heavy metals are classic developmental neurotoxicants, having long been known to induce a variety of effects on the central nervous system. Since reports of major incidents of mercury-related toxicity in Minimata Bay, Japan, in the 1950s[145], and in Iraq in the 1970s[146], methylmercury (MeHg) neurotoxicity has been well studied[145]. Although there are naturally occurring sources of mercury (Hg) (e.g., soil and rock erosion), human activity represents a large portion of mercury contamination in the environment[147]. Organic forms of mercury, such as MeHg, are formed from inorganic mercury, converted through the process of methylation by aquatic microorganisms in water and sediment[148]. The presence of MeHg in fish, particularly in predatory fish due to biomagnification, represents a primary source of human exposure[149], particularly in populations whose main diet consists of fish and seafood[150]. Thus, similar to PFAAs, dietary intake of MeHg represents the main route of human exposure.

In humans, once ingested, MeHg is efficiently absorbed in the gastrointestinal tract, and distribution of MeHg occurs through passive diffusion or from forming a complex with sulfhydryl (R-SH) containing molecules (e.g., L-cysteine)[151]. The MeHg-cysteine complex is then distributed throughout the body by neutral amino acid transporters[151,152], allowing efficient transfer across the blood/brain barrier[153] and the placenta[154]. The elimination of MeHg in humans includes a reaction with glutathione within in the liver, and similar to PFOS, MeHg is excreted though bile[147]. However, unlike PFOS, which undergoes enterohepatic circulation, much of MeHg and inorganic Hg can be eliminated in feces by this route, resulting in a much shorter half-life in humans (e.g., MeHg half-life ~44 days)[155,156].

1.5.2 Experimental Models of MeHg Neurotoxicity

MeHg exposure in animal models has shown diverse neurological implications, particularly if exposure occurs during pregnancy[157–159]. Single high-dose MeHg exposure during gestation or lactation induces learning impairments and behavioural modifications[160–164] that have been linked to changes in gene[160] and protein[165] expression, altered neurochemical signaling pathways (e.g., glutamate)[161], and changes in brain morphology and neuron cell density[164,166–169]. Apart from single high doses, effects from chronic low-level exposure (i.e.,

exposure to MeHg in contaminated drinking water) in maternal dams induces subtle effects on offspring including deficits in sensory, motor, learning and tests of memory[158,170,171], sometimes occurring without any observable changes to brain morphology[165].

Chronic low-dose MeHg in the presence of other environmental contaminants represents a realistic human exposure scenario. It has been hypothesized that the effects of MeHg are exacerbated when co-exposed with contaminants that share common exposure sources, including PFAAs[172], polybrominated diphenyl ethers (PBDEs)[173], and PCBs[174–179]. Combined exposure to MeHg and PBDEs had an additive effect, inducing deficits in reflexes and motor coordination of newborns, whereas combined MeHg and PCBs was antagonistic, inducing effects from either MeHg or PCBs individually were not observed with co-exposure to both compounds simultaneously.

There are only a small number of experimental investigations of PFAAs and their effects on neurodevelopment (see previous Section 1.3.4), and even fewer that include co-exposure with other contaminants. To my knowledge, only one study in rats, conducted by Cheng et al. investigated the combined effects of perinatal co-exposure to MeHg and PFAAs[172]. In that study, combined exposure to MeHg and PFOA induced development deficits in the reflexes of newborn rat pups, whereas motor function testing in older juveniles revealed antagonistic effects[172]. However, female rats are not ideal model organisms in which to test PFOA toxicity, whose active elimination of PFOA results in a short 2-hour half-life, compared to 5.5 days in male rats and 16 days in mice[180]. Female rats exhibit increased expression and activity of organic anion transporters (OATs), allowing PFOA to be preferentially eliminated by renal clearance and substantially lowering its body burden[181], resulting in less transfer to the developing fetus.

In Chapter 2 I designed an experiment to test the developmental toxicity of perinatal exposure to PFOS and/or MeHg to address the limited data on developmental toxicity of PFAAs as chemical mixtures. Unlike PFOA, the half-life of PFOS in female rats was approximately 60 days following a single oral dose[84], which was long enough to ensure sufficient fetal transfer and exposure. Furthermore, a low-dose and a high-dose of PFOS with MeHg co-exposure was included to examine the dose-dependent response of PFOS with MeHg.

1.6 Maternal PFAA Exposure and Thyroid Hormone Status During Pregnancy

Maintaining hormone homeostasis is important due to their involvement in numerous physiological processes, including metabolism, cardiac function, and mental status[182]. Regulation of the hypothalamic-pituitary-thyroid (HPT) axis occurs through the inhibitory action of thyroid hormones and stimulatory action of thyrotropin-releasing hormone (depicted in Figure 1-3). A detailed explanation of thyroid hormone regulation and feedback is described in Mariotti and Beck-Peccoz[183]. In brief, low levels of circulating thyroid hormones stimulate the production and release of thyrotropin-releasing hormone (TRH) from the hypothalamus. TRH induces synthesis and secretion of thyroid stimulating hormone (TSH) from the anterior pituitary gland which, in turn, stimulates the production and release of thyroxine (T4) and triiodothyronine (T3) from the thyroid gland. Although lesser amounts of T3 are released from the thyroid, most T3 in circulation is the product of deiodination in peripheral organs (e.g., kidney and liver)[183]. The increased levels of T3 and T4 in circulation independently inhibit the production of more TRH.

1.6.1 PFAAs Disrupt Fetal Thyroid Development

Thyroid hormones are also essential for healthy fetal development during pregnancy, playing an important role in brain maturation, and are involved in the regulatory processes of neurogenesis, neuronal migration, proliferation, and myelination[184–186]. Onset of fetal thyroid function does not occur until between 16 and 20 weeks gestation[187], with demonstrable fetal serum concentrations of T3 and T4, from production in fetal thyroid follicles, that continually increases throughout gestation[188]. Thus, the fetus relies entirely on a maternal supply of thyroid hormones throughout pregnancy, but with increased demand in early pregnancy prior to initiation of fetal thyroid function. This exerts an additional stress on maternal thyroid production, and so, endocrine disruptors that would normally be compensated for in the general population may be more potent when exposure occurs during pregnancy.

PFAAs have been considered endocrine disrupting contaminants that are recognized to affect thyroid hormone regulation and metabolism[189]. In rats, direct PFAA exposure has been linked to decreased testosterone and increased estradiol concentrations[190]. In humans, increased occupational exposure to PFOA in fluorochemical workers was negatively associated with free T4 (FT4), and positively associated with T3[191]. According to NHANES data, increased PFOA

and PFOS concentrations in men and women in the US are linked to increased prevalence of thyroid disease[192]. According to the US EPA, both PFOS and PFOA were listed in their Endocrine Disruptor Screening Program in 2013, establishing the need for a range of tests that contribute to the weight of evidence that these contaminants are involved in endocrine activity and whether that activity leads to adverse outcomes[193]. The presence of thyroid disrupting contaminants in the gestational environment, such as PFAAs, may compound the stress on maternal thyroid hormone production, transport or metabolism during sensitive stages of pregnancy[185,189].

1.6.2 Experimental Models of PFAA-Induced Thyroid Hormone Disruption

In rodent developmental toxicology studies, PFOS generally elicits effects on thyroid hormone metabolism that are consistent with hypothyroxinemia[100,101,130,194], defined as low FT4 (and/or total T4) without any compensatory increase in TSH. Women are typically considered hypothyroxinemic if categorized in the lowest 10th percentile of FT4 while in the normal range of TSH for a given sample population. In monkeys dosed with either PFOS[92] or PFOA[195], T3 and T4 homeostasis can be altered. However, literature may not be reliable at high doses of PFOS and PFOA because measurements of T4 using analog methods were prone to negative bias in the presence of PFOS, which was absent in gold-standard methods (i.e., equilibrium dialysis followed by radioimmunoassay) (detailed in Chang et al.[196]).

In humans, a comparison of FT4 measurements using either analog or dialysis did not show differences across a range of PFOS and PFOA levels in serum, indicating that the observed bias in animals may not be relevant for human studies. Human T4 transport proteins include thyroid binding globulin (TBG), transthyretin (TTR), and albumin, whereas rats do not have TBG and are primarily reliant on albumin and TTR[197]. Since most PFAAs have a stronger binding affinity for TTR than for TBG, animals may be more susceptible than humans to thyroid dysfunction from PFAA exposure[198,199]. Despite these limitations, experimental models with rodents provide strong indications that PFAAs can affect thyroid physiology by disrupting the HPT-axis (Figure 1-3), justifying investigations of PFAA-induced thyroid dysregulation in humans.

1.6.3 Epidemiological Findings on PFAAs and Maternal Thyroid Status

A recent meta-analysis of PFAA exposure and adult thyroid hormone status in the general population (i.e., non-pregnant) found that PFOS was positively correlated with FT4 and negatively correlated with TT4 and TT3, and both PFOA and PFHxS were negatively correlated with TT4[200]. Establishing consistent patterns of association between PFAA exposure and thyroid hormone status in pregnancy cohorts can be difficult, as outcomes may be influenced by differences in experimental design[201]. Nevertheless, the most consistent finding among these studies is a positive association between PFAA and TSH[202–205], suggesting that PFAA exposure may be a risk factor for maternal hypothyroidism. Associations of PFAAs with maternal T3 and T4 during pregnancy have been less consistent. For example, using 1st trimester samples, Preston et al. found that increased levels of PFOA and PFHxS were negatively associated with FT4[206], whereas in Wang et al. 3rd trimester samples showed that other PFAAs (PFNA, PFUnA, and PFDoA) were negatively associated with both FT4 and TT4[204], but the same was not true for PFOA nor PFHxS. Moreover, Berg et al. found none of these PFAAs were significantly associated with maternal FT4 or TT4 in an investigation where samples were collected in the 2nd trimester[202]. Such findings demonstrate the time-dependency of PFAA associations with thyroid hormones during pregnancy.

A comparison of numerous birth cohort studies that examined maternal thyroid homeostasis during pregnancy indicated differences in PFAA exposure levels between countries at different years of collection (Table 1-3). The US[206] and Canada[205] had higher maternal levels of PFHxS, whereas PFNA and PFUnA were higher in maternal samples from Taiwan[204]. The US and Taiwan also had among the highest maternal levels of PFOS, but this may be confounded by the early collection time for these cohorts (between 1999 and 2002, prior to the major phase-out)[204,206]. PFAAs are highly correlated across each trimester[207], thus single sample collections can provide robust measurements of maternal PFAA exposure. However, as discussed above, pressure on maternal thyroid production is not constant throughout gestation, and thyroid hormones change gradually over gestation. The time of sample collection during pregnancy may explain the discrepancies between findings between these investigations[201].

The presence of additional stressors during pregnancy (e.g., thyroid conditions) may also influence associations between PFAAs and maternal thyroid hormones. In a recent Canadian

birth cohort, PFOS was positively associated with TSH and negatively associated with FT4, but only in a subset of women who tested positive for thyroid peroxidase antibodies (TPOAb)[205], a marker of autoimmune thyroiditis (Hashimoto's disease). Thus, subsets of pregnant TPOAb positive women may be more susceptible to PFAA-associated thyroid hormone dysregulation than otherwise healthy pregnant women.

PFAA exposure on maternal thyroid hormones may be further influenced by the presence of other endocrine disruptors. As previously discussed, both PFAAs and Hg share similar sources of exposure, primarily through dietary intake. After exposure, both contaminants are distributed throughout circulation, with PFAAs predominantly present in plasma, and Hg in red blood cells. During pregnancy, PFAA exposure affects levels of FT4, generally leading to a condition of hypothyroxinemia[100,101,130,194]. Hg has also shown a propensity for endocrine disruption in past cohort studies, and was negatively associated with T3 during pregnancy[208,209] and in 6-month old infants[210]. PFAA-thyroid hormone disruption is hypothesized to be from competitive inhibition of T4 binding to serum proteins[198]. Hg is considered to have a more direct influence on the HPT-axis, affecting the activity of deiodinase enzymes, with type III deiodinases being the most sensitive to disruption[211]. Monoiodothyronine deiodinase (types, I, II, and III) are enzymes involved in the activation and deactivation of specific thyroid hormones. It was suggested that deiodinase enzyme inhibition during pregnancy would alter production of T3 from T4, that ultimately result in offspring neurobehavioural changes[212]. Thus, during pregnancy, thyroid hormone regulation via the HPT-axis, critical to fetal development, provides a common mechanism for adverse neurodevelopment from PFAA and/or Hg exposure.

In Chapter 3 I address many of these hypotheses in a highly detailed longitudinal study of a Canadian birth cohort, whereby multiple observations of maternal thyroid hormones (including TPOAb) are made during pregnancy (trimester-specific), as well as post-pregnancy. Longitudinal observations of thyroid hormones allow for the consideration of the influence of time, and significant time-dependent associations in this chapter will be specifically identified. I also consider other stressors, including TPOAb and interaction with total Hg (THg) exposure, and I measure the exposure to PFAAs with a highly resolved isomer-specific method. Although it is well established that organic mercury (MeHg) makes up the bulk of human mercury from dietary exposure[213], all reported blood concentrations of mercury in human samples from Chapter 3 refer to the reported analysis of THg. To my knowledge, previous studies have not accounted for

modified associations from co-exposure with other environmental contaminants (e.g., THg) or include a detailed analysis of branched PFOA and PFOS isomers.

1.7 Epidemiology Studies of PFAAs and Neurodevelopment

As previously discussed (Section 2.3), temporal trends of major PFAAs (PFOS and PFOA) show declining concentrations, but lesser-studied PFAAs (PFHxS and PFNA) have remained the same or may have increased[75,214]. With the exception of some hot spots (e.g., high PFOA levels from contaminated drinking water in the mid-Ohio river valley[215], or high PFBS and PFHxS levels in Uppsala, Sweden[216]), PFAAs are frequently detected at relatively low exposure levels across numerous birth cohorts.

According to recent reviews (Roth and Wilks[217], and Liew et al.[218]), only a limited number of studies have examined neurodevelopmental and neurobehavioural consequences of perinatal PFAA exposure. The majority of these studies have focused on physical birth outcomes and early childhood outcomes, finding various adverse associations of prenatal and postnatal PFAA exposure with body weight and length, ponderal index (a calculated measure of leanness), as well as with abdominal and head circumference[218]. A few studies have investigated the implications of PFAA exposure on early neurodevelopment, from as early as 5 weeks up to 2 years, and findings have been mixed. For example, in Japan, PFOA was inversely associated with Bayley Scales of Infant and Toddler Development at 6 months but not at 18 months[219]; in Taiwan, PFOS, but not PFOA, was adversely associated with developmental indices of gross motor subdomains in 2 year olds[220]; neither PFOS nor PFOA affected the development of newborns or infants in Denmark[221] or the US[222]. When monitoring exposure and development at these early stages (< 2 years old), only PFOS and PFOA concentrations were considered, and no other PFAA analytes, nor their corresponding isomers, were included in these investigations.

In older school-age children and adolescents (ages 4 to 18), initial cross-sectional studies identified links between PFAA exposure and increased incidence of ADHD and impulsivity[223–225]. However, these findings were not reinforced by longitudinal cohort studies[226–228], with no associations for PFOA nor PFOS with increased risk of ADHD or impulsivity, even within one of the largest birth cohorts to include PFAA exposure (the Danish National Birth Cohort, DNBC) with >83,000 participants[226]. Furthermore, numerous investigations in the US[229], Denmark[230], the

Faroe Islands[231], as well as Greenland, Ukraine, and Poland[232] found no association between prenatal exposure to PFOA, PFOS, PFNA, or PFDA with any behavioural outcomes. Regarding prenatal PFAA exposure and child cognitive development, there is a paucity of data available, and findings are inconsistent. For example, Vuong et al. reported PFOA-associated deficits in executive function of children[233], and Harris et al. found that multiple PFAAs (PFOA, PFOS, and PFHxS) were associated with decreased visual-motor development[234]. However, in an extensive battery of tests evaluating child IQ (reading, math, language, and memory testing), none of these PFAAs had significant associations[235,236].

These findings indicate the potential for maternal PFAA exposure to affect child behavioural and cognitive development, but contrasting outcomes in the literature warrant the need for more well-designed studies to confirm the impact of specific PFAAs on child development[217,218]. In Chapter 4, I examine the association of prenatal PFAA exposure on neurological development of 2-year-old children in the APrON cohort using outcomes from standardized testing (Bayley Scales of Infant and Toddler Development, 3rd Edition (Bayley-III)). Isomer-specific PFOS and PFOA associations and interaction with THg co-exposure were also considered. THg is reported in Chapter 4 the same manner as in Chapter 3, as total mercury content.

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Table 1-1. Names, chain length, formulas, and acronyms of PFAAs

Class	Compound	Chain length	Formula	abbreviation
Perfluoroalkyl Carboxylates				
	Perfluorobutanoate	4	$C_3F_7CO_2^-$	PFBA
	Perfluoropentanoate	5	$C_4F_9CO_2^-$	PFPA
	Perfluorohexanoate	6	$C_5F_{11}CO_2^-$	PFHxA
	Perfluoroheptanoate	7	$C_6F_{13}CO_2^-$	PFHpA
	Perfluorooctanoate	8	$C_7F_{15}CO_2^-$	PFOA
	Perfluorononanoate	9	$C_8F_{17}CO_2^-$	PFNA
	Perfluorodecanoate	10	$C_9F_{19}CO_2^-$	PFDA
	Perfluoroundecanoate	11	$C_{10}F_{21}CO_2^-$	PFUnA
	Perfluorododecanoate	12	$C_{11}F_{23}CO_2^-$	PFDoA
	Perfluorotridecanoate	13	$C_{12}F_{25}CO_2^-$	PFTrA
	Perfluorotetradecanoate	14	$C_{13}F_{27}CO_2^-$	PFTeA
Perfluoroalkyl Sulfonates				
	Perfluorobutane Sulfonate	4	$C_4F_9SO_3^-$	PFBS
	Perfluorohexane Sulfonate	6	$C_6F_{13}SO_3^-$	PFHxS
	Perfluorooctane Sulfonate	8	$C_8F_{17}SO_3^-$	PFOS
	Perfluorodecane Sulfonate	10	$C_{10}F_{21}SO_3^-$	PFDS

Table 1-2. Names and acronyms of PFOA and PFOS branched isomers

Class	Compound	abbreviation
Isomers of PFOA		
	L-Perfluorooctanoate	L-PFOA
	6m-Perfluorooctanoate	6*m*(*iso*)-PFOA
	5m-Perfluorooctanoate	5*m*-PFOA
	4m-Perfluorooctanoate	4*m*-PFOA
	3m-Perfluorooctanoate	3*m*-PFOA
	∑dm-Perfluorooctanoate	∑*dm*-PFOA
Isomers of PFOS		
	L-Perfluorooctane Sulfonate	L-PFOS
	6m-Perfluorooctane Sulfonate	6*m(iso)*-PFOS
	5m-Perfluorooctane Sulfonate	5*m*-PFOS
	∑4m+3m-Perfluorooctane Sulfonate	∑4*m*+3*m*-PFOS
	1m-Perfluorooctane Sulfonate	1*m*-PFOS
	∑dimethyl-Perfluoroactane Sulfonate	∑*dm*-PFOS

Table 1-3. Frequency of detection (%), median concentration and range (ng/mL) of detected PFAAs from recent cohort studies

Country (size)	Collection (year)	Collection	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFHxS	PFOS
Canada[205]	2007-2008	2nd trimester	99 %	62 %	-	-	.	84 %	100 %
(n = 152)			1.7	0.6	-	-	.	1.0	4.8
			(1.0 - 2.4)	(<0.5 - 0.8)				(0.7 - 1.7)	(3.2 - 6.5)
USA[206]	1999-2002	1st trimester	99-100 %	99-100 %	-	-	.	99-100 %	99-100 %
(n = 726)			5.6	0.6	-	-	.	2.4	24
			(3.9 - 7.7)	(0.5 - 0.8)				(1.6 - 3.8)	(17.6 - 32.6)
Korea[113]	2008-2009	3rd trimester	100 %	84 %	93 %	61 %	.	100 %	100 %
(n = 44)			1.46	0.44	0.31	0.60	.	0.55	2.93
			(1.15 - 1.91)	(0.23 - 0.62)	(0.24 - 0.39)	(0.50 - 0.99)		(0.46 - 0.85)	(2.08 - 4.36)
China[237]	2013	Delivery	100 %	100	100 %	99 %	68 %	100 %	100 %
(n = 157)			1.64	0.46	0.37	0.40	0.04	0.5	4.41
			(0.73 - 8.11)	(0.13 - 1.69)	(0.04 - 2.07)	(<LOD - 1.27)	(<LOD - 0.15)	(0.12 - 4.22)	(0.73 - 8.11)
Taiwan[204]	2000-2001	3rd trimester	87 %	96 %	71 %	91 %	82 %	78 %	100 %
(n = 285)			2.39	1.51	0.46	3.26	0.36	0.81	12.7
			(1.54 - 5.20)	(0.85 - 6.20)	(0.1 - 1.09)	(1.70 - 22.05)	(0.23 - 0.85)	(0.30 - 2.90)	(9.65 - 27.9)
Norway[202]	2007-2009	2nd trimester	100 %	100 %	-	-	-	99 %	> 80 %
(n = 391)			1.53	0.56	-	-	-	0.44	8.03
			(0.99 - 2.16)	(0.43 - 0.78)				(0.28 - 0.66)	(5.76 - 11.0)
Japan[238]	2002-2005	1st/2nd trimester	100 %	-	-	-	-	-	100 %
(n = 392)			1.2	-	-	-	-	-	5.2
			(<LOD - 3.4)						(1.6 - 12.3)

- Value was not recorded, PFAA not considered

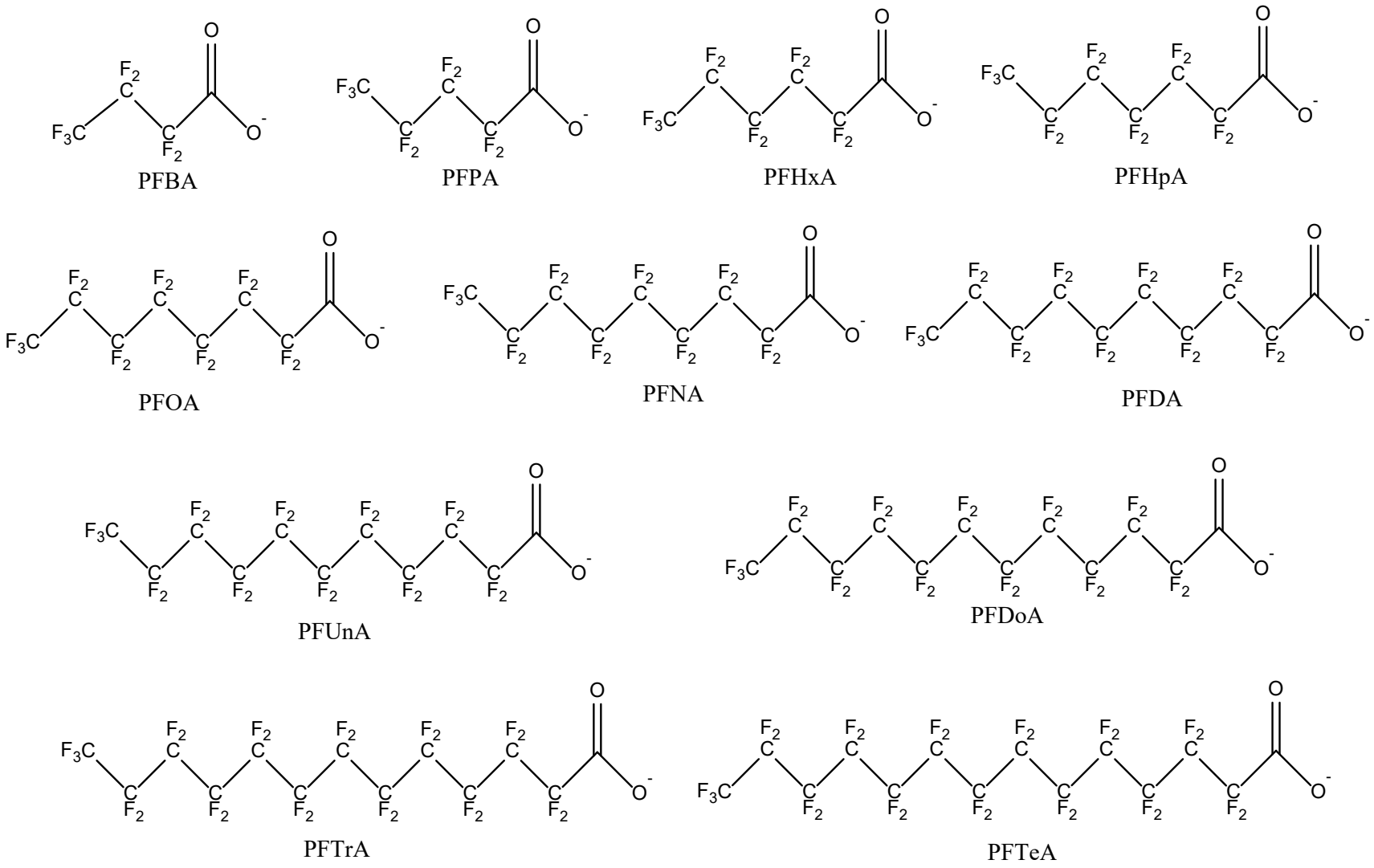
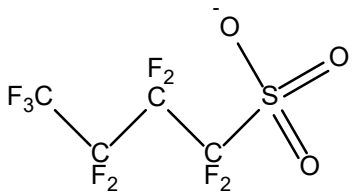
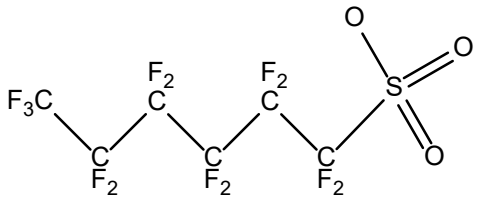


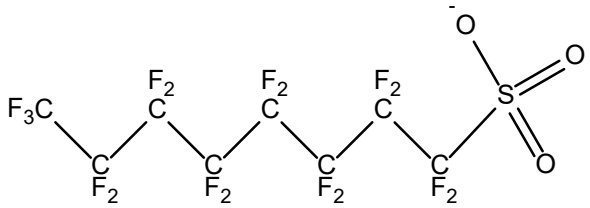
Figure 1-1A. Structures of perfluoroalkyl carboxylates 4 to 14 carbons in chain length included in PFAA analysis.



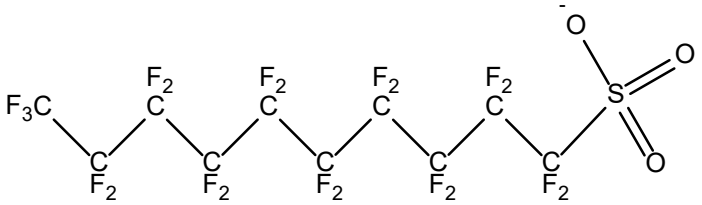
PFBS



PFHxS



PFOS



PFDS

Figure 1-1B. Structures of perfluoroalkyl sulfonates 4, 6, 8, and 10 carbons in chain length included in PFAA analysis.

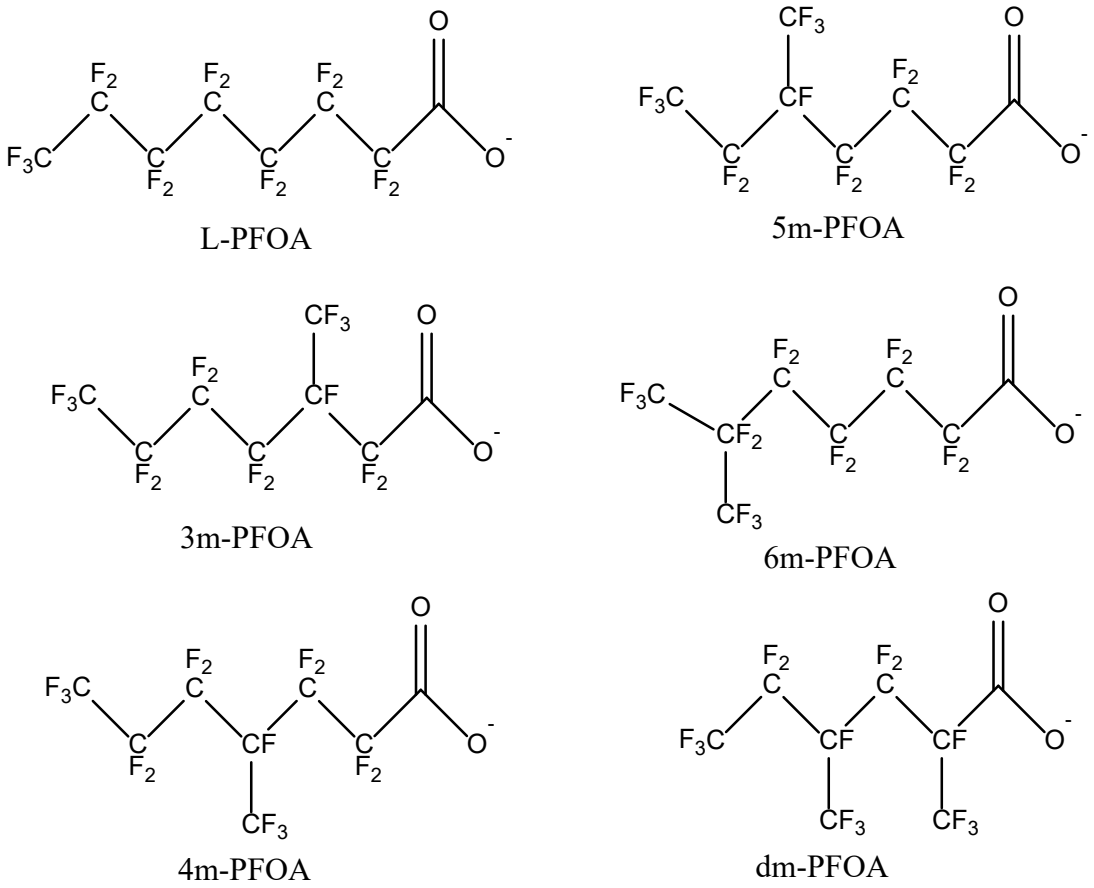


Figure 1-2A. Structures of branched isomers of PFOA. An example structure of dimethyl PFOA (dm-PFOA) is shown, there is the potential for other variations.

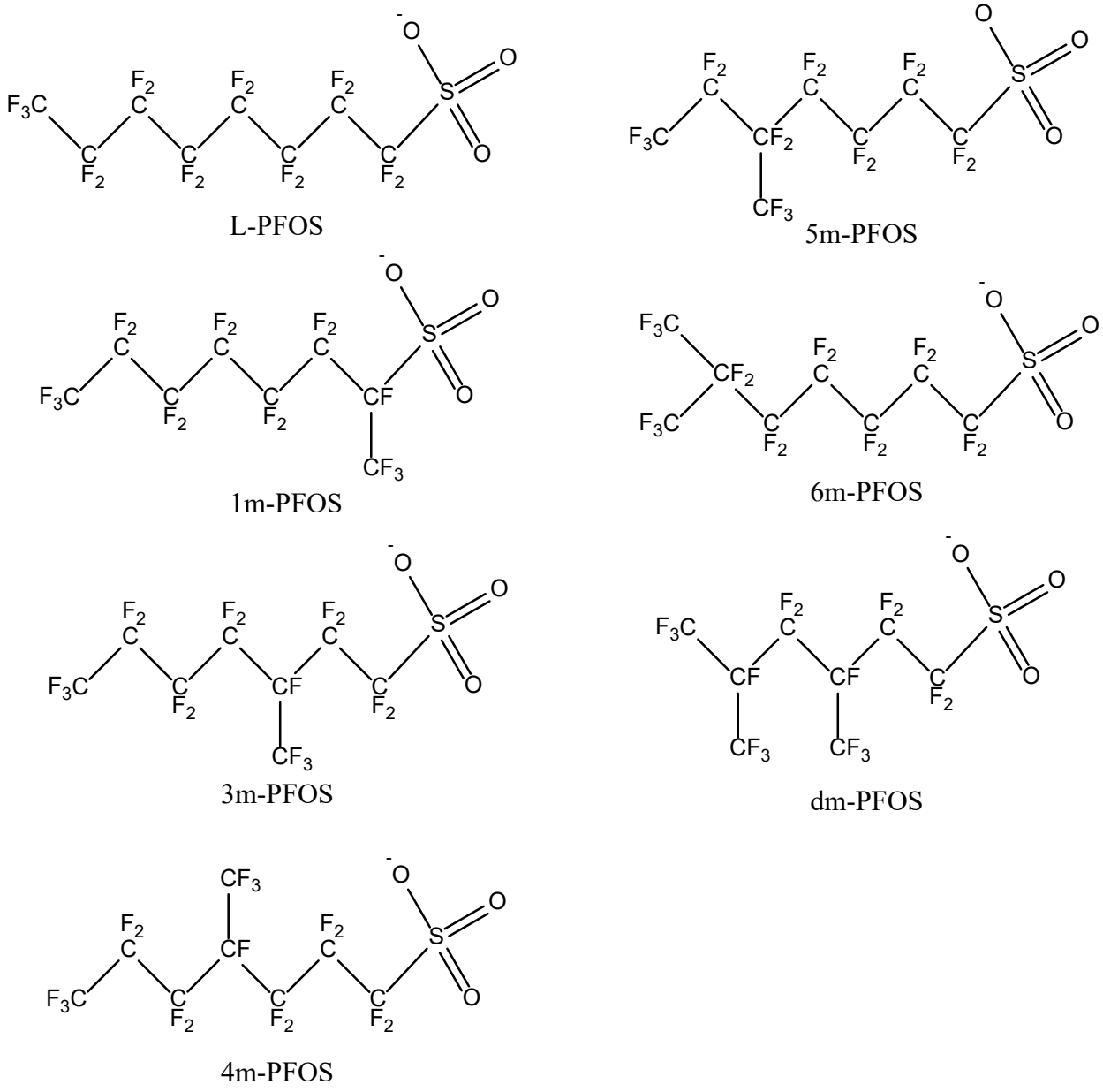


Figure 1-2B. Structures of branched isomers of PFOS. An example structure of dimethyl PFOS (dm-PFOS) is shown, there is the potential for other variations.

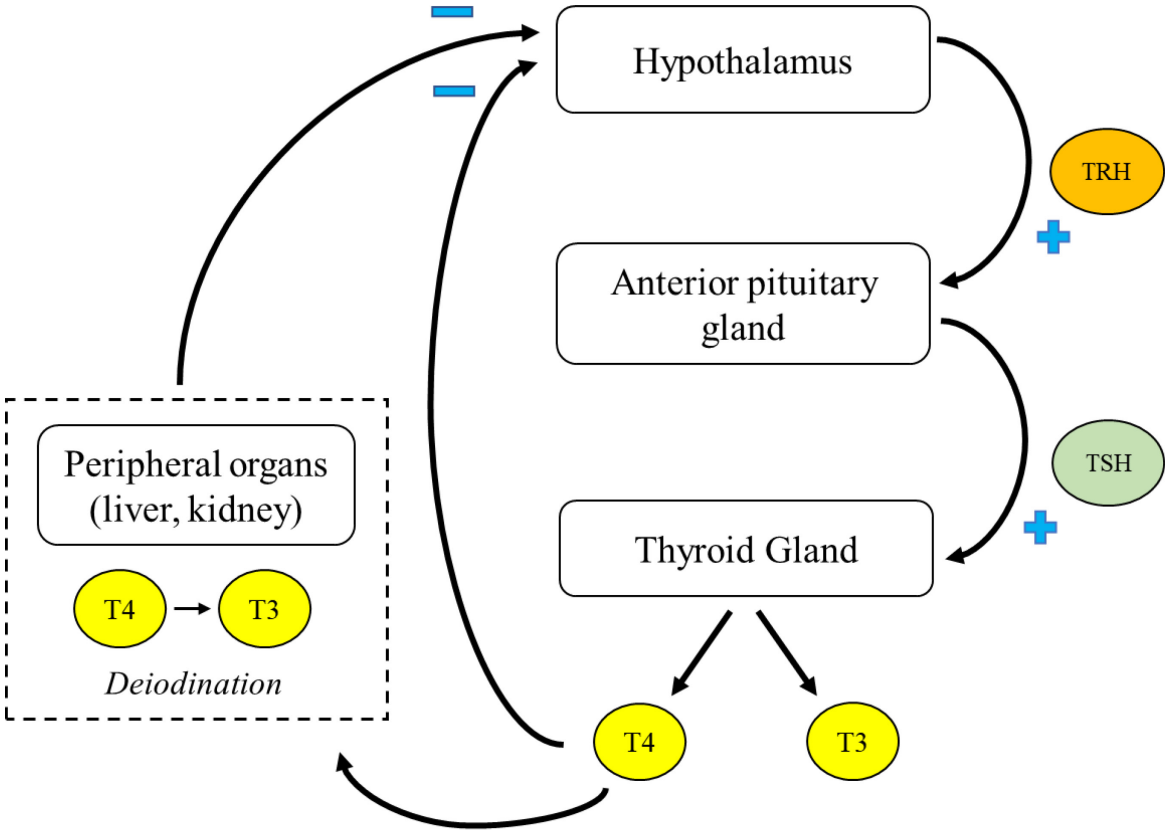


Figure 1-3. The basic elements of the HPT-axis in regulation of thyroid hormone synthesis. The plus sign indicates stimulation, and the negative sign indicates inhibition.

Chapter 2

Neurodevelopmental and Metabolomic Responses from Prenatal Co-Exposure to Perfluorooctane Sulfonate (PFOS) and Methylmercury (MeHg) in Rats

2.1 Introduction

Perfluorooctane sulfonate (PFOS) and methylmercury (MeHg) are pervasive environmental contaminants. PFOS is among many environmental perfluoroalkyl acids (PFAAs) that have been used directly as surfactants or are the degradation products of precursor substances used in surface treatment of textiles and food packaging. The widespread presence of PFOS has been attributed to its long historical use, environmental stability and persistence, and lengthy blood elimination half-life in people (~5.4 yrs)[1]. Although PFOS levels in human blood have declined significantly in North America and Europe since production was discontinued by its major manufacturer (3M company)[2,3], it remains the predominant organic contaminant in human blood[4,5]. In addition to the voluntary production phase-out, the human health concerns over PFOS and its precursors has led to their addition to Annex B of the *Stockholm Convention on Persistent Organic Pollutants* in 2009[6].

MeHg, a potent neurotoxicant[7], is produced naturally through biological methylation of mercury and progressively bioaccumulates up the aquatic food chain[8]. Emission of total Hg, and estimated MeHg levels exported from ocean fisheries have steadily increased since the 1950s[9]. Although mercury has a relatively short half-life in humans (~ 44 days)[10,11], chronic MeHg exposure has led to greater blood concentrations in populations with sources of increased exposure (i.e., living near tropical artisanal gold mining sites, in the Arctic regions that includes a diet of apex predators, and in the Pacific and Mediterranean coastal regions whose diet includes commercially sourced seafood)[12]. Dietary intake represents a major route of human exposure to both PFOS[13–15] and MeHg[16] and populations whose diet include a major proportion of fish and seafood have higher blood levels of PFOS[17–19] and MeHg[20].

During pregnancy, both PFOS[21] and MeHg[22,23] can efficiently cross the placenta into fetal circulation. Total PFOS placental transfer efficiency (i.e., ratio of concentration in cord blood to maternal blood) from past cohort studies has ranged from (0.36 – 0.48)[24–26]. However, branched isomers of PFOS tend to have higher transfer efficiencies (up to 0.88)[27,28], and

comparatively, Hg placental transfer is even higher (mean = 1.86)[22]. Toxicokinetic investigations in rats have demonstrated differences in uptake and elimination of linear and branched isomers of PFOS[29,30] that raises questions about isomer-specific toxicity of PFOS during pregnancy.

Individually, both MeHg and PFOS have each been associated with deficits in neurodevelopment. Animal models of developmental toxicity demonstrated both behavioural and molecular effects in offspring as a result of maternal MeHg exposure (see reviews by Johansson et al.[31], Castoldi et al.[32] and Bisen-Hersh et al.[33]). In brief, gestational exposure results in offspring learning and memory deficits[34–37], and decreased motor function[38,39]. These MeHg-induced changes in neurobehavioural outcomes in animals were linked to altered brain morphology and neuron density[35,40–43], as well as modified gene[44] and protein[45,46] expression. The neurodevelopmental effects of PFOS have received less attention, but chronic low-dose exposure to PFOS in pregnant rats altered open-field behavior[47,48] and motor function[49,50] in offspring compared to controls. Evidence is also mounting in humans, with studies examining associations of PFAAs (including PFOS) with fetal growth indicators[51–53], and motor and mental development milestones in infants[54]. Cross sectional studies have reported associations between PFAAs and increased incidence of attention deficit/hyperactivity disorder (ADHD) and impulsivity in school-age children[55,56].

Although MeHg and PFOS act independently as developmental neurotoxicants and share common sources of human exposure (e.g., dietary intake), their combined effects have never been investigated. The effects of individual contaminants within environmental mixtures have difficult to interpret in epidemiological models[57], thus experimental animal models are valuable tools for testing mixtures, and their effects, whether independent, additive, synergistic or antagonistic. MeHg has been investigated in conjunction with persistent organic pollutants with common exposure sources, including both polychlorinated biphenyls (PCBs) and organochlorines (OCs)[58–63]. However, only one study to date has investigated the effects from co-exposure to both MeHg and PFAAs using perfluorooctanoate (PFOA)[46].

In this study, dietary co-exposure mixture effects of MeHg and PFOS were investigated in a developmental model using pregnant Sprague-Dawley rats. Objectives were to determine if a mixture of PFOS and MeHg elicits different effects than corresponding individual chemical exposures, and to determine if the mixture effect changed with a low- or high- doses of PFOS.

Growth and development were monitored in offspring and behavioural outcomes were examined at both the pre-weaning and juvenile stages. Brain metabolomic profiles were used to identify changes in levels of specific target metabolites (lipids, biogenic amines, amino acids, etc.,) in the brain that may be linked to observed changes in behaviour. Metabolomics involves a comprehensive analysis of the metabolites within a biological system to reveal alterations of biochemical pathways using analytical tools and instrumentation[64]. Targeted metabolomics have been utilized in developmental toxicity models of fish[65,66] and mice[67], identifying changes in metabolites from exposure to PFAAs and other environmental contaminants that may be involved in underlying mechanisms of altered behaviour.

2.2 Methods[I]

2.2.1 Animal Treatment and Dosing

All protocols and procedures were approved by the Animal Care and Use Committee (animal use protocol, AUP #809) at the University of Alberta in compliance with guidelines of the Canadian Council on Animal Care and the Animal Protection Act (Government of Alberta, revised 2000). Sprague-Dawley rats (25 females and 12 males) were obtained from Charles River Laboratories (Laval, Quebec) and acclimated for 3 weeks prior to mating. Animals were subjected to a 12 hr light/dark cycle and had unlimited access to food and water. Animals were bred by placing two females and one male overnight in a cage, after which pregnancy was confirmed by the appearance of a vaginal plug with the presence of sperm, denoted as gestational day (GD) 0. At GD 1, pregnant dams were randomly assigned to one of five treatment groups, and fed commercial gelatin (strawberry-flavoured Jello®) containing either MeHg, PFOS, combinations of both chemicals, or untreated Jello® (control). At birth, pups were culled to 8 animals/litter (5 female and 3 male), denoted as postnatal day (PND) 0. Dosing of maternal dams continued until weaning at PND 21.

During pregnancy, dams were dosed according to treatment group. A stock solution of technical-grade PFOS (Wellington Laboratories, Guelph ON) was prepared by dissolving the

[I] I was responsible for experimental design, ethics approval, and project oversight, including: conducting animal dosing, behavioural tests, tissue collection, and chemical analysis.

chemical in a 75:25 mixture of water and reagent alcohol (Sigma-Aldrich). Methylmercury chloride (CH_3HgCl, Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich. Stock solutions were diluted approximately 100-fold in Jello® prior to feeding individual pregnant rats from a plastic cup, with the amount adjusted based on rat body weight. The treatments were: Control (gelatin containing vectors from treatment groups), PFOS-only (1 mg PFOS/kg/d), MeHg-only (1 mg MeHg/kg/d), Low-Mix: (1 mg MeHg/kg/d + 0.1 mg PFOS/kg/d), and High-Mix (1 mg MeHg/kg/d + 1 mg PFOS/kg/d). The dose levels of MeHg and PFOS were selected based on previously published reports of the lowest observed adverse effect level (LOAEL) for offspring in chronic exposure studies of MeHg[46,68–70] and PFOS[49,71,72], to avoid maternal toxicity and neonatal mortality.

2.2.2 Analysis of Mercury[II]

A 100 µL aliquot of blood was diluted (20, 100, and 5000x) in volumes of LC/MS grade water. Samples were diluted further with a basic solution containing 25 µg/L of iridium internal standard, 10 µg/L gold, 0.5 g of EDTA in 1% v/v ammonia hydroxide, 2.5% butanol, and 0.05% v/v Triton X100. Treatment groups containing MeHg had basic solutions that were 2-fold more concentrated. The final diluents were then analyzed for total mercury by inductively coupled plasma mass spectrometry (ICP-MS/MS). QA/QC data from mercury analysis is available in Appendix B.

2.2.3 Analysis of PFOS

PFOS was analyzed in the serum fraction of maternal and offspring whole blood samples. Samples were highly concentrated and diluted in LC-MS grade water (10, 100, 1000 and 10,000×) to the appropriate range for instrument detection. Diluted serum was extracted using a protein precipitation method modified from Glynn et al.[73]. Briefly, aliquots (0.5 mL) of each dilution containing an isotopically labeled PFOS standard (MPFAC-MXA; Wellington Laboratories) were extracted using 4 mL of acetonitrile (ACN). Samples were sonicated for 10 min in a room temperature water bath, followed by 5 min centrifugation at 2000 rpm (Eppendorf

[II] Dr. Amy MacDonald and Dr. David Kinniburgh from the Alberta Centre for Toxicology, Calgary, Canada conducted mercury analysis

Sorvall ST-40R tabletop centrifuge, Thermo-Fisher Scientific). The supernatant was transferred to a 15 mL tube and evaporated in a 40 °C water bath under nitrogen gas to a volume of 0.2 mL, after which the extract was reconstituted to 1 mL in a 50:50 mixture of methanol and water. The diluted extract underwent dispersive cleanup[74], transferring the extract to a 1.7 mL Eppendorf tube containing approximately 0.025g of bulk graphitized carbon (Supelclean ENVI-Carb, Sigma Aldrich), that had been acidified with 50 µL of glacial acetic acid and mixed by vortex for 10 sec. The sample was centrifuged for 10 min at 10,000 rpm (Sorvall Legend Micro 21R, Thermo Scientific) and the top 0.5 mL was transferred to an auto-sample vial.

Isomer-specific analysis of PFOS with HPLC-MS/MS was performed according to the method described in Benskin et al.[75] using a UFLC-XR Shimadzu HPLC coupled to an API 5000 triple quadrupole mass spectrometer (Applied Biosystems Sciex, Concord, ON). Branched PFOS was calculated as the sum of individual branched isomers (*iso*-, 5*m*-, 3*m*+4*m*-, 1*m*-, and ∑*dimethyl*-PFOS), quantified from an external solvent-based calibration curve spiked with native PFOS standard (BrPFOSK, Wellington Laboratories). Method validation procedures and results of recovery experimentation are presented in Appendix A.

2.2.4 Growth Rate of Dams and Reproductive Outcomes

Various reproductive outcomes, including: pregnancies carried to term, length of pregnancy, and litter size were monitored during the dosing period to assess reproductive success (Table 2-1). The weight of dams was recorded every 3 days during gestation (GD 1 to GD 21) and lactation (PND 2 to PND 21).

2.2.5 Pre-Wean Observations and Testing of Newborn Rat Pups[III]

Every two days from PND 3 until PND 21, three pups were randomly selected from each litter of each group for observation and testing (Table 2-2). A modified version of the Fox Test Battery was used to observe physical development and evaluate the reflexes of newborn rat pups[76]. Prior to reflex testing, each pup was weighed, and monitored for developmental markers, including incisor eruption, hair growth, pinnae detachment, ear opening, and eye opening. Test responses were scored as follows: righting reflex: the recorded time for the rat to place all four

[III] Jacqueline Karathra assisted with offspring neurodevelopment testing.

paws on a surface when initially placed on its back (cutoff time of 2 sec); cliff drop aversion: the recorded time for the rat to retract its head and forepaws when placed on the edge of a tabletop with forepaws and head over the edge (cutoff time of 5 sec); negative geotaxis: the recorded time to turn 180º when originally placed with head pointing downward on a 45º slope (cutoff time of 5 sec).

2.2.6 Offspring Post-Wean Behaviour[IV]

Post-wean tests (Table 2-2) were initiated at PND 35 using 2 randomly selected females from each exposure group. Prior to testing, group labels were concealed and randomized, and tests were conducted by researchers that were blind to exposure groups. An isolated test-space was established to minimize visual and auditory cues, and all testing was conducted under uniform conditions of light (30 lux) and temperature (22 ºC). The open-field arena and all mazes were built of black plexiglass (2 cm thickness) to track animal activity by contrast (white rat on black background), that was recorded on an overhead camera and analyzed using Ethovision XT motion tracking software (v10, Noldus Information Technology, VA, USA).

2.2.6.1 Open Field Arena

The software designated border and corner zones that were superimposed over the entire arena (100 × 80 × 30 cm height). Rat pups were allowed to freely explore for a trial time of 5 min over two consecutive trials that were conducted with each rat, using a 24 hr inter-trial interval. Open-field activity was monitored, including: total distance traveled (cm), velocity (cm/sec), frequency of zone crossings (counts), in-zone duration (sec), and latency to first entry of each zone (sec). The frequency (counts) and duration (sec) of rearing activity were also observed.

[IV] Dr. Karim Fouad from the Department of Physical Therapy (University of Alberta) assisted with project design for animal behaviour testing and provided the facilities and apparatus necessary for testing.

Dr. Trevor Hamilton Department of Psychology (Grant MacEwan University) provided the camera and animal tracking software.

2.2.6.2 Rotating Rod (Rotarod)

Offspring were tested on a single-lane commercial rotating rod apparatus (Med Associates, St. Albins, VT). A modification was applied to smooth the grooves of the axle (treadmill lane) to reduce the ability of the rats to cling to the rod and increased the difficulty of the task[77,78]. Rats were first subjected to a habituation phase 1 hour prior to testing for 2 min at 4 rpm. The testing phase included single trial exposure accelerating (from 4 to 40 rpm) over 5 min[79].

2.2.6.3 Elevated Plus Maze

A commercial plus maze (Med Associates, St. Albins, VT) elevated at a height of 75 cm, consisted of two perpendicular open platforms (50 × 10 cm) and two perpendicular closed arms (50 × 10 × 40 cm) branching from a centre junction (12 × 12 cm). All rats were placed facing the same direction at the centre junction between the open and closed arms and allowed 5 min for maze exploration. The total distance (cm), velocity (cm/sec), as well as the frequency (counts) and duration (sec) of arm entries (open and closed) was recorded. An index of anxiety-like activity was calculated, defined as the proportion of time spent in open arms divided by the total maze time.

2.2.6.4 Novel Object Recognition

The novel object recognition test was modified from Ennaceur and Delacour[80]. Rats were previously habituated to the testing environment, as testing took place 24-hrs after the open field test. The objects to be discriminated were yellow rubber ducks and black and white penguins. Multiple sets of objects were utilized and cleaned between trials to eliminate bias from olfactory stimuli, and separate naïve animals that were not involved in behaviour testing were used to verify that rats did not display preference for either test object.

Testing consisted of two trials separated by an inter-trial time of 1 hour. The first trial (familiarization), allowed the animals to freely explore two identical objects placed in opposite corners of the arena for 10 min. A second trial (choice) replaced one of the familiar objects with a novel object and allowed animal exploration for 2 min. Exploration activity was defined as the animals' head direction toward the object with nose proximity < 2 cm. The time of head

direction facing toward, but not within proximity of the object was also recorded. The frequency (counts), investigating duration (sec), and latency to approach (sec) either familiar or novel objects was recorded in addition to total exploration time. Object exploration and discrimination indices were calculated using formulas from May et al.[81] (refer to Table 2-3).

2.2.6.5 Radial Arm Maze

The radial arm maze consisted of a centre junction (30 cm in diameter) with 8 arms of equivalent dimensions (40 × 10 × 30 cm) branching out from the centre point. Food restrictions were implemented to increase rat motivation for rewards (banana flavoured pellets, 45 mg) during testing. Rats were habituated to the radial arm maze 24-hrs prior to training, each animal was given 5 min to explore an unbaited maze. The training phase with all arms baited was conducted for consecutive days until rats were able to navigate the maze without revisiting any target arms. The testing phase only baited 4 target arms and left 4 non-target arms (unbaited). Rats could freely explore the maze until all target arms were visited (maze completion) or until a threshold of 5 min. The testing was repeated for 3 consecutive days, recording the following parameters: reference errors (non-target arm visits), working memory errors (target arm revisits), and total errors.

2.2.7 Tissue Preparation, Metabolite Extraction and Analysis for Metabolomics[V]

Whole brains were removed immediately after euthanization and snap-frozen by submerging in isopentane kept on dry ice. The time between extraction and snap-freezing was kept constant for all rats to minimize post-mortem associated fluctuations in metabolite levels. Frozen brains were kept on dry ice until partially thawed, before dissecting brainstem, cerebellum, hypothalamus, hippocampus and cortical sections. Sectioned brain samples were placed in 15 mL polypropylene centrifuge tubes and stored at -80 ºC.

[V] I performed tissue extraction and analysis using methods and facilities provided by Anton Ribbenstedt and Dr. Jonathan Benskin in the Department of Environmental Science and Analytical Chemistry (University of Stockholm, Sweden). Anton Ribbenstedt performed consolidation and deconvolution of data for PCA and PLS-DA modelling.

Extractions were carried out by addition of solvent (1:4 chloroform and methanol) using 5 µL per mg tissue. Smaller brain sections (hippocampus) were diluted at 10 µL solvent/mg tissue ensure a sufficient extraction volume for later analysis. The solvent/tissue mixtures underwent disruption with beads using a 1600 MiniG automated tissue homogenizer (SPEX Sample Prep®) for 2 min at 1500 rpm (×2 intervals) using zirconium beads for soft tissue (hippocampus, hypothalamus, cortex), and steel beads for dense tissue (brainstem, cerebellum). Homogenates were centrifuged at 3000 RCF for 5 min in a tabletop centrifuge, after which the supernatant was removed and underwent ultracentrifugation at 12,000 RCF, transferring the top 0.5 mL of extract to a 2.4 mL Eppendorf tube. An autosample vial with a final volume of 300 µL consisted of 10 µL of sample, 60 µL of lipid internal standard, 20 µL of internal standard for targeted analysis of amino acids and biogenic amines and 210 µL of methanol.

The targeted metabolomics method measures up to 199 metabolites, including, lipids, amino acids (AAs) and biogenic amines (BAs), by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) with quantification by authentic standards. Determination of lipids, including acylcarnitines (ACs), ceramides (CERs), sphingomyelins (SMs), phosphotidylcholines (PCs), and lyso-phosphotidylcholines (lysoPCs) was carried out using flow injection tandem-mass spectrometry with isotopic overlap deconvolution together with a limited number of authentic standards. Nomenclature for amino acids and biogenic amines utilized a standard 3-letter notation (e.g., alanine = ala) while lipids were named according to Ribbenstedt et al.[82]. Briefly, glycerophospholipids were defined based on the presence of ester and/or ether bonds (represented by an ‘a’ or ‘e’, respectively), a ‘C’ denoting the fatty acid, followed by chain length and the number of double bonds separated by a colon. Two letters (ae = acyl-alkyl, aa = diacyl) denote fatty acids bound to two glycerol positions (e.g., PCaaC20:2). Sphingomyelin (SM) nomenclature uses the same nomenclature but with a ‘d’ to denote the backbone sphingosine (e.g., SM (d18:0/C18:1). Carnitines are denoted by a ‘’ followed by the corresponding number of carbons and double bonds separated by a colon (e.g., C2:0). A complete list of analytes, internal standards, and corresponding nomenclature for metabolomic analysis is found in Appendix A.

2.2.8 Data Handling and Statistical Analysis

Statistical analysis of newborn and juvenile development outcomes was conducted with IBM SPSS (version 24.0). Due to smaller samples sizes (n = 4 to 5 litters), and non-linearity of data, non-parametric tests were conducted for all pre-wean and post-wean data. A preliminary Kruskal-Wallis test was used to evaluate the differences across all treatment groups (level of significance = 0.05). If considered to be significant, a group comparison was conducted to determine which groups were significantly different (level of significance p = 0.05). In order to reduce the potential for type-1 error, a Bonferroni correction was applied by dividing the level of significance by the number of group comparisons.

Statistical analyses of metabolomics data was carried out with Metaboanalyst 3.0 (www.metaboanalyst.ca)[83]. Analytes below the lowest level of detection or above the highest level of quantification in brain section subgroups (e.g., cortex) were not included in multivariate statistical analysis. Kruskal-Wallis testing identified significant ($p < 0.05$) target metabolites between exposure groups within each dissected brain subsection. Principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were applied to data normalized with auto scaling (mean centered for each variable). PCA and PLS-DA scores plots indicated one outlier from each of the Control and Low-Mix groups, separate from the rest of the cluster within each treatment group. Outliers were confirmed with the outlier detection function of Random Forest testing within Metaboanalyst, and outliers were removed in final PCA and PLS-DA models. Cross-validation of PLS-DA models and permutation testing producing R2, and Q2 values and levels of significance are found in Appendix A. Hierarchal clustering of heatmap data used the Ward clustering algorithm and Euclidean distance measure. The pathway analysis feature of Metaboanalyst using the KEGG database identified specific pathways affected by PFOS and/or MeHg exposure. Pathway analysis uses a reference metabolome and provides the "pathway impact" if altered metabolites are in key positions of the pathway network, and a "logP" indicating the level of significance of exposure induced deviations of metabolite concentrations from controls.

In offspring, brain region-specific metabolite profiles were compared among controls, PFOS-only, MeHg-only, Low-Mix and High-Mix. Of 199 metabolites, 147, 136, 127, 132, and 138 were in the range of detection for brainstem, cerebellum, cortex, hippocampus and

hypothalamus, respectively. Only the cortical metabolomic profiles were considered, as the cortex was the only tissue subsection that showed significant differences between treatment groups.

2.3 Results and Discussion

2.3.1 Maternal and Offspring Exposure to PFOS and MeHg

Chemical analysis of plasma PFOS and blood total mercury of dams and offspring was conducted with samples collected after weaning (3 weeks post-birth). PFOS plasma concentrations of dams and offspring in the High-Mix group were similar to the PFOS-only group, and higher than the Low-Mix group. As expected, blood mercury content was consistent across all MeHg exposure groups (Figs. 2-1A and B). For both PFOS and Hg, significantly lower concentrations were observed in offspring compared to dams (5-fold and 40-fold lower, for PFOS and Hg respectively). The maternal-offspring transfer efficiency of PFOS was greater than for MeHg (i.e., comparing maternal High-Mix to offspring High-Mix groups), yet both can cross the placenta. Animal models have indicated a trend of decreasing blood and brain mercury during the lactation period[68], and a similar trend has also been established in humans during the first few months of breastfeeding[84,85]. Breastmilk represents a significant source of PFOS excretion for lactating mothers[86] but contains low amounts of Hg that is primarily present in the inorganic form[87,88]. The combination of decreased transfer efficiency of MeHg during lactation, coinciding with increasing blood volumes of rapidly growing newborn rat pups may explain decreased Hg blood levels in offspring.

The ratio of linear to branched PFOS isomers (%) in maternal plasma decreased when compared to the ratio in offspring (Fig. 2-2). Transplacental transfer efficiency was further influenced by the structure of PFOS, with branched isomers of PFOS enriched in offspring plasma, an observation that was consistent with previous human studies[24,27,28]. A proposed mechanism by Beesoon et al. using in-vitro models suggested that the linear conformation of PFOS binds more tightly to serum proteins, leaving unbound branched PFOS isomers freely available to cross the placenta[89]. In rats, uptake, distribution and excretion rates of PFOS vary based on structure[29,30], but isomer-specific PFOS consequences on the fetus from exposure in utero are largely unknown. Although it is beyond the scope of the current investigation, the

observed increase in fetal exposure of offspring to branched isomers of PFOS warrants further study into isomer-specific differences in the developmental toxicity of organic contaminants.

2.3.2 Maternal Growth and Reproduction

There were no differences in gestation length or number of pups between exposure groups and no overt complications in pregnancy were noted (Table 2-1). Survival rates of pups were 100 % in all litters. Maternal growth during gestation (GD0 to GD19), and lactation (PD 1 to PD 21) also did not show any treatment effect (Fig. 2-3A).

2.3.3 Newborn Offspring Growth and Reflex Development

Growth of pups (Fig. 2-3B) and pre-wean reflex testing (Fig. 2-4) showed significant delays in development of the combined exposure groups. Offspring weight gain was significantly decreased in both combined exposure groups (Low-Mix and High-Mix, $p < 0.05$), whereas PFOS-only and MeHg-only rats were not different from controls. Nevertheless, there were no differences in offspring achievement in any of the developmental markers (listed, Table 2-2). There were no significant treatment differences in the proportion of newborns able to complete reflex ontogeny tasks (righting reflex, cliff avoidance, or negative geotaxis (Fig. 2-4, left column). However, a noticeable floor effect was observed for both cliff aversion and negative geotaxis (Fig. 2-4B and C, respectively) up to PND 9, and a ceiling effect for both tasks during the last week (> PND 15). Therefore, we restricted comparisons to a specific period in the neurodevelopment window (Fig 2-4, right column) and tests of righting reflex and negative geotaxis showed significant delayed response times among High-Mix pups compared to controls ($p < 0.05$). In the cliff aversion task, High-Mix pups were significantly delayed compared to control, PFOS-only, and MeHg-only on Day 11 ($p < 0.05$) (Fig. 2-4B).

The pre-wean stage of newborn rats represents a sensitive stage of early neurological development that has been identified as the brain growth spurt, a period of rapid brain growth occurring postnatally for rodents (PND 0-7) and in utero for humans (late 3rd trimester)[90]. The postnatal onset in rodents enables observation of behavioural effects during stages of synaptogenesis, gliogenesis and myelination that are not readily observed in humans[91]. Combined exposure (High-Mix) decreased growth rates and induced delayed reflex responses in newborn rats that did not occur in either PFOS-only or MeHg-only treatment groups (Figs. 2-3,

and 2-4). Decreased growth and significantly delayed development of offspring has been observed in previous developmental toxicity studies of PFOS alone, but only at dosing levels that coincided with increased neonatal mortally[71]. Current results indicate that chronic exposure to PFOS and MeHg caused developmental delays in newborns without inducing mortality.

In previous co-exposure studies of MeHg with PFOA[46], or MeHg with PCBs[58], both individual and combined exposure scenarios induced development delays, and made it difficult to determine if a toxicological interaction was taking place. However, the current data show an interaction on newborn development, whereby significant effects were observed, but only from combined exposure to both PFOS and MeHg (High-Mix).

2.3.4 Post-Wean Behaviour

PFOS-only offspring were more active in the open field arena, with significantly increased travel distance, and velocity (Fig. 2-5A and B, respectively) compared to controls and the High-Mix group ($p < 0.05$). A visual representation of activity as heat maps (Fig. 2-5D) confirmed increased activity in the PFOS-only treatment. Offspring from both PFOS-only and MeHg-only treatments also had increased frequency of border crossings (Fig. 2-5C). Although PFOS-only and MeHg-only induced changes in offspring behaviour, these effects were absent in both combined exposure groups, suggesting an antagonistic toxicological interaction. PFOS-only offspring also performed better on the accelerating rotarod (Fig. 2-6), with significantly increase time to fall ($p < 0.05$) compared to the control and High-Mix groups.

PFOS-only offspring displayed hyperactivity in the open field (Fig. 2-5) that has been observed in previous studies of PFOS developmental toxicity in rats[92], mice[47,48], and zebrafish[93,94]. In zebrafish larvae, the PFOS-induced increase in activity was negated by administration of dopamine receptor agonists or amphetamine-like psychostimulants (e.g., dexafetamine, prescribed for treatment of ADHD), suggesting that the hyperactivity originates from altered levels of neurotransmitters[93]. PFOS-only rats also had significant increases in "time to fall" on the rotarod ($p <0.05$, Fig. 2-6), a routinely administered test of motor coordination in rats and mice that was suggestive of improved motor function from PFOS exposure. However, outcomes on the rotarod may be influenced by the physical (i.e., level of activity), or mental (i.e., level of anxiety) state of the rat during testing. For example, mice administered low doses of

ethanol also increased their time to fall on the rotarod, but higher ethanol doses ultimately resulted in decreased time to fall (reduced performance)[95]. Similar to low-dose ethanol, PFOS may have a physical stimulatory effect, increasing rat activity that manifests as increased time spent on an accelerating rotarod. When considered in conjunction with increased travel distance and velocity in the open field, the increased time to fall of rats on the rotarod apparatus was more likely attributed to PFOS induced hyperactivity rather than improved motor function.

In the elevated plus maze, High-Mix offspring had an increased number of entries (frequency), and a greater duration of the test time spent in open platforms when compared to controls (Fig. 2-7), resulting in an increased anxiety-index ($p < 0.05$), calculated as the proportion of time spent in open arms over the total maze time. An increased index represents an increased anxiolytic response (i.e., decreased fear with an observed increased willingness to explore novelty). Further analysis of combined exposure groups revealed PFOS-dose-dependent changes in frequency, duration, and calculated index, as High-Mix offspring were significantly different from controls and Low-Mix offspring (Fig. 2-7B). A visual representation of location preference and mean activity as heatmaps (Fig. 2-7C) showed control animals spent less time on open platforms (blue outline) and more time in closed arms (red outline) compared to both combined exposure groups (Low-Mix and High-Mix). Thus, increasing exposure to PFOS in the presence of MeHg corresponds with an increased anxiolytic activity on the elevated plus maze.

In the open-field MeHg-only rats had increased border crossings, but unlike PFOS-only, this did not coincide with increased distance or velocity, and instead may be attributed to increased thigmotaxic behaviour (Fig. 2-5). Thigmotaxis is described as part of the rats natural defense, avoiding predation by staying near to vertical surfaces[96], a response that has been linked to anxiety-like behaviour[97]. Thus, MeHg-only offspring had increased border crossings but stayed in close proximity to walls of the open field arena, representative of a thigmotaxic response and increased anxiety-like behaviour. However, MeHg-only did not alter offspring behaviour on the elevated plus-maze (Fig. 2-7). Furthermore, increasing PFOS in the presence of MeHg from combined exposure (Low-Mix and High-Mix) reduced anxiety-like behaviour in a dose-dependent manner, as these rats more freely explored the open platforms of the elevated maze. Taking these findings into account suggests maternal exposure to PFOS or MeHg alone is not enough to alter anxiety-related behaviour, but the combination of both contaminants with increasing PFOS had an interacting cumulative effect. To our knowledge this is the only study to

demonstrate a dose-dependent change in anxiety-related behavior from combined exposure to an organic contaminant with a heavy metal. Considering the anxiolytic nature of the observed response on the elevated plus maze, decreased exploration in the open field may be due to MeHg-related impairments in motor function. Although our findings suggested that offspring motor function on the rotarod was affected by prenatal exposure to MeHg (Fig. 2-6), a finding that was not significant after application of the Bonferroni correction, but is supported by past studies of MeHg exposure related decreases in rat motor function[59,98,99], specifically decreased time to fall on the rotarod[38,45,46,68].

Offspring underwent two types of memory testing, a 1-day object recognition test of non-spatial oriented memory (Table 2-4) and a multi-day test of spatial memory using the radial arm maze (Table 2-5). None of the treatment groups were different from controls in either object recognition testing or performance in the radial arm maze, findings that agree with past studies of repeated low-dose exposure to prenatal MeHg exposure in rodents[36,45,46,58,100,101]. In humans there is evidence of cognitive impairment from prenatal exposure to MeHg, whereas rodent models have inconsistent outcomes from cognitive testing[32,33]. However, the rat body burden of Hg during post-wean stages may not be sufficient to induce cognitive deficits, even when combined with exposure to additional contaminants, such as PFOS.

2.3.5 Altered Metabolomic Profiles

All dissected brain regions (cortex, cerebellum, hippocampus, hypothalamus and brainstem underwent metabolomic analysis, and were found to have distinct metabolite profiles between brain regions (Appendix A). However, only offspring rat cortex had distinct differences in profiles between treatment groups, with 68 alterations from a total of 127 detectable metabolites (Fig. 2-8). The observed antagonism at the behavioural level from combined exposure to PFOS and MeHg was reinforced by metabolomic data. For example, metabolite profile clusters in PLS-DA scores plot of individual chemical exposure groups (PFOS-only and MeHg-only) were distinct from controls, but both combined exposure groups (Low-Mix and High-Mix) overlapped with controls (Fig. 2-8A). The majority of variance (~54 %) between treatments were explained by the first two components of the model, with the majority on the x-axis (component 1) accounting for 47.6 %. The loadings plot identified the lipids (primarily

phosphatidylcholines) and specific amino acids that primarily contributed to treatment separation in the PLS-DA scores plot (Fig. 2-8B).

An unsupervised hierarchal cluster analysis (heatmap) portrayed the extent of change in metabolite response (rows) between individual treatment groups (columns) (Fig. 2-8C). A distinct cluster was observed for PFOS-only and MeHg-only, and another cluster for control and both combined exposure groups. For significantly altered levels of amino acids, higher concentrations were observed in the exposure to MeHg-only or PFOS-only compared to either the control or combined exposure groups. The opposite trend was observed in lipids, with higher concentrations observed in control and combined exposure groups compared to MeHg-only or PFOS-only groups (Fig. 2-8C).

Perturbation of lipids (primarily phosphatidylcholines and sphingomyelins) levels in the MeHg-only and PFOS-only treatment groups are consistent with previous findings for PFOA[67]. Phosphatidylcholines within lipid membranes are considered reservoirs for choline, a precursor to acetylcholine, one of the primary neurotransmitters of the cholinergic system[102]. In mice, hyperactivity from exposure to PFOA and PFOS was altered by injections of nicotine[48]. Numerous investigations have also shown the capacity of PFAAs to affect expression of the alpha isotype of peroxisome proliferator activated receptors (PPARs)[103]. PPARα, primarily expressed in the liver, heart and skeletal muscle, plays a key role in lipid metabolism[104]. However, PPARα is also strongly expressed in the prefrontal cortex of rodents and humans[105]. It is reasonable then, to postulate that exposure to PFOS, and to a lesser extent MeHg, affects lipid metabolism through altered PPARα expression, leading to decreased concentrations of PCs and disruption of cholinergic neurotransmission that ultimately impact behavioural outcomes.

Levels of specific amino acids and neurotransmitters (green dashed circle) influenced the separation of clusters in the scores plot (Fig. 2-8A). Increased concentrations of GABA, taurine, Gly, Met, Pro, Ser, and T4-hydroxyPro with PFOS-only, and increased Thr and Ser levels from MeHg-only exposure were observed compared to controls ($p < 0.05$) (Fig. 2-9). Metabolic pathways involving Gly, Ser, thr, Ala, Asp, Arg, Pro, and Glu, were significantly affected by individual exposure to PFOS or MeHg but there were no significant changes observed in either combined exposure group. These changes in levels of amino acids and neurotransmitters and their subsequent effects on metabolic pathways indicate target neurotransmission systems that

are susceptible to change from exposure to PFOS or MeHg that may be linked to corresponding changes in behaviour.

Specific metabolites are involved in a variety and physiological processes and provide potential targets of neurotoxicity from PFOS and MeHg exposure. Neurotransmitters GABA and Gly (Fig. 2-9), and their corresponding metabolic pathways (Fig. 2-10) were significantly affected by exposure to PFOS or MeHg. MeHg alters the activity of GABAα receptors, blocking GABAergic synaptic responses, inducing a hyper-excitability of neurons and increasing susceptibility to seizures[106]. In addition to GABA, neonatal exposure to MeHg alters locomotor activity mediated by the dopaminergic system[107]; as dopamine D2 receptors in the striatum of rats were reduced from MeHg exposure, ultimately decreasing locomotor activity, an effect remedied by D2 receptor agonists[98,99]. PFOS alters the concentration and gene expression of both D1 and D2 dopaminergic receptors in prefrontal cortex, hippocampus and amygdala brain[108]. However, dopamine levels were below detection limits in the current study, and we were unable to observe differences between treatment groups for either dopamine or serotonin. However, Asp, Gly, and Ser, agonists of N-methyl-D-aspartate (NMDA) receptors, were significantly elevated from PFOS exposure (Fig. 2-9). NMDA, a glutamate receptor that interacts with dopamine receptors, modulates a variety of cognitive and motor functions in the brain[109]. Increased excitatory neurotransmission from activation of NMDA receptors provides a basis for increased spontaneous activity in PFOS-exposed rats, as well as providing an explanation for concomitant feedback increases of inhibitory neurotransmitters such as GABA.

2.4 Summary and Conclusions

Behavioural outcomes of offspring prenatally to PFOS, MeHg, or combined exposure are summarized (Table 2-6). Behavioural outcomes at different development stages revealed differences in the extent of toxicological insult from in utero co-exposure. Combined exposure to PFOS and MeHg significantly affected the growth and development of newborn rat pups, effects that were not observed from PFOS or MeHg alone. Previous animal studies of animal co-exposure had difficulty determining if significant interactions were taking place[46,58], whereas we observed interacting effects from combined exposure to PFOS and MeHg (High-Mix). The difference in significance between our findings and past studies may be due to differences in exposure timing, dosage level, and method of contaminant delivery. Therefore, further studies

are warranted to determine the extent of effects from individual and combined exposure to environmental contaminants at very early stages of neurological development. In older juveniles, PFOS and MeHg alone altered activity of rats in the open field and on the rotating rod, not present in either combined exposure group. Comparison of metabolomic profiles revealed similar patterns of an antagonistic chemical interaction. However, the observed antagonism in older juveniles may not be an indication of recovery from the initial effects that were observed in newborn rats, as permanent changes induced from toxicological insult may not be readily observed when testing during adolescence or adulthood.

Our own observations of chemical antagonism were consistent with past studies of MeHg with PFOA[46], and PCBs[58,60–62], where it has been suggested that organic contaminants have the capacity to mask effects of MeHg. For example, Coccini et al. found that relatively high doses of PCB 153 affected the influence of MeHg on cholinergic muscarinic receptors given that both compounds have different molecular targets it was suspected that combined exposure to "PCB153 may cause conformational changes in MeHg binding sites, preventing it from exerting its effect"[61]. In our own findings, effects from combined exposure revealed in early reflex testing of newborns were likely not recovered from or nullified in older juveniles but more likely masked or compensated for by neuronal plasticity.

The addition of multiple chemicals from various exposure sources contribute to what I describe as an exposure bubble. Continual additional low-dose exposure to various chemical mixtures during critical times of fetal development will eventually lead the bubble to burst, eliciting permanent and irreversible changes in neurological function.

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Table 2-1. List of reproductive outcomes among pregnant dams exposed to individual (PFOS-only and MeHg-only) or combined (Low-Mix or High-Mix) exposure groups

	Control (n = 5)	**MeHg** (n = 4)	**PFOS** (n = 4)	**Low-Mix** (n = 4)	**High-Mix** (n = 4)
Gestation (days)	21 ± 0.0	21.3 ± 0.3	21 ± 0.0	21 ± 0.0	21 ± 0.0
Litter size (#)	13.0 ± 1.0	11.5 ± 1.0	12.5 ± 1.3	12.0 ± 1.3	11.3 ± 1.3

values are presented as the mean ± SE

There were no significant differences from group comparisons ($p < 0.05$)

Table 2-2. Timing of pre- and post-wean observations and testing for pups and juveniles

Parameter	**Post-Natal Day (PND)**
Growth	3 – 19
Incisor eruption	3 – 19
Hair growth	3 – 19
Eyelid opening	3 – 19
Pinnae detachment	3 – 19
Ear opening	3 – 19
Righting reflex	3 – 19
Cliff drop aversion	3 – 19
Negative geotaxis	3 – 19
Open Field	38 – 40
Rotating Rod	41 – 42
Novel object recognition	43 – 44
Elevated plus maze	45 – 46
Radial arm maze	53 – 61

Table 2-3. Formulas for calculation of object exploration and discrimination indices from novel object recognition testing

Variables	Exploration	Discrimination
A_1 (exploration of object 1, trial 1)	$E_{T1} = A_1 + A_2$	$D_1 = A_3 - B$
A_2 (exploration of object 2, trial 1)	$E_{T2} = A_3 + B$	$D_2 = D_1/E_{T2}$
A_3 (exploration of familiar object, trial 2)		$D_3 = A_3/E_{T2}$
B (exploration of novel object, trial 2)		

Variables were used to calculate the total object exploration time (sec) of trial 1 (E_{T1}), and total object exploration time (sec) of trial 2 (E_{T2}). Discrimination indices of absolute time (sec) of novel object discrimination (D_1) and the ratio of discrimination of novel (D_2) and familiar (D_3) objects

Table 2-4. Calculated exploration and discrimination indices from novel object recognition testing

	Control (n = 8)	**PFOS-only** (n = 8)	**MeHg-only** (n = 8)	**Low-Mix** (n = 8)	**High-Mix** (n = 8)
Duration					
T1 Exploration (sec)	4.4 ± 5.1	8.9 ± 5.2	9.9 ± 7.9	3.9 ± 7.1	5.6 ±6.4
T2 Exploration (sec)	17.0 ± 2.5	17.7 ± 2.3	21.4 ± 1.9	14.2 ± 2.3	18.1 ± 5.6
Discrimination (sec)	4.4 ± 1.4	8.9 ± 1.7	9.9 ± 2.9	3.9 ± 1.2	5.6 ± 1.3
D2 Index	0.26 ± 0.01	0.49 ± 0.06	0.45 ± 0.12	0.31 ± 0.11	0.33 ± 0.07
D3 Index	0.55 ± 0.05	0.40 ± 0.09	0.38 ± 0.09	0.52 ± 0.08	0.38 ± 0.06
Frequency					
T1 Exploration (counts)	2.1 ± 2.7	1.8 ± 3.3	4.5 ± 4.9	2.9 ± 5.6	2.3 ± 5.0
T2 Exploration (counts)	11.1 ± 1.1	12.0 ± 1.7	11.8 ± 1.4	12.4 ± 1.7	10.8 ± 1.0
Discrimination (counts)	2.1 ± 0.7	1.8 ± 0.5	4.5 ± 0.7	2.9 ± 0.4	2.3 ± 0.2
D2 Index	0.19 ± 0.05	0.14 ± 0.04	0.44 ± 0.10	0.28 ± 0.04	0.22 ± 0.06
D3 Index	0.47 ± 0.04	0.49 ± 0.03	0.47 ± 0.10	0.61 ± 0.04	0.47 ± 0.05

Values are represented as the group mean ± SE

*$p < 0.05$, level of significance, no groups were significantly different based on Kruskal-Wallis and Mann-Whitney non-parametric group comparisons (refer to table 2-2 for the index calculation formulas)

Table 2-5. Performance from testing trials on the radial arm maze

	Trial	Control (n = 8)	PFOS-only (n = 8)	MeHg-only (n = 8)	Low-Mix (n = 8)	High-Mix (n = 8)
Task completion (sec)	1	27.4 ± 3.50	29.2 ± 8.00	28.8 ± 5.60	42.5 ± 15.2	53.6 ± 20.2
	2	36.8 ± 13.6	31.2 ± 6.70	72.0 ± 38.3	39.4 ± 6.70	37.7 ± 6.8
	3	26.7 ± 5.10	73.3 ± 31.8	108 ± 42.6	43.8 ± 10.9	26.5 ± 10.4
Total errors (#) [A]	1	3.4 ± 0.4	3.7 ± 0.8	3.3 ± 0.8	4.0 ± 0.7	5.5 ± 1.8
	2	3.4 ± 0.7	5.1 ± 1.3	5.1 ± 1.7	5.0 ± 0.9	4.5 ± 0.8
	3	3.4 ± 0.7	8.2 ± 2.6	7.3 ± 2.8	5.3 ± 1.0	3.0 ± 0.7
Working memory errors (#) [B]	1	0.3 ± 0.2	1.0 ± 0.4	0.7 ± 0.4	0.7 ± 0.4	2.3 ± 1.6
	2	0.8 ± 0.4	1.8 ± 1.1	1.9 ± 1.5	1.5 ± 0.6	1.2 ± 0.5
	3	0.6 ± 0.3	5.3 ± 2.3	4.7 ± 2.8	2.2 ± 0.9	0.7 ± 0.5

Values are represented as the group mean ± SE

*$p < 0.05$, level of significance, no groups were significantly different based on Kruskal-Wallis and Mann-Whitney non-parametric group comparisons

[A] total error: the sum of revisiting target arms (baited) after first visit, and non-target (unbaited) first visit and revisits

[B] working memory error: target (baited) arm and non-target (unbaited) revisits only

Table 2-6. Summary table of behavioural outcomes in rat offspring prenatally exposed to individual or combined PFOS and/or MeHg

Apparatus and Test Parameters		PFOS-only	MeHg-only	Low-Mix	High-Mix
Pre-wean testing (newborns)					
	growth weight	-	-	↓	↓
	righting reflex	-	-	-	↓
	cliff aversion	-	-	-	↓
	negative geotaxis	-	-	-	↓
Post-wean testing (juveniles)					
Open-Field	distance travelled	↑	-	-	-
	velocity	↑	-	-	-
	border crossing	↑	↑	-	-
Rotating Rod	performance	↑	-	-	-
Elevated Plus Maze	anxiety Index	-	-	↑[A]	↑[A]
Novel Object Recognition	non-spatial memory	-	-	-	-
Radial Arm Maze	spatial-oriented memory	-	-	-	-

Outcomes represented as significantly ($p < 0.05$) increased (↑) or decreased/delayed (↓) response
Black arrows correspond to changes in comparison to controls, and red arrows to changes from both control and High-mix treatment groups.

[A] response was observed to be dose-dependent

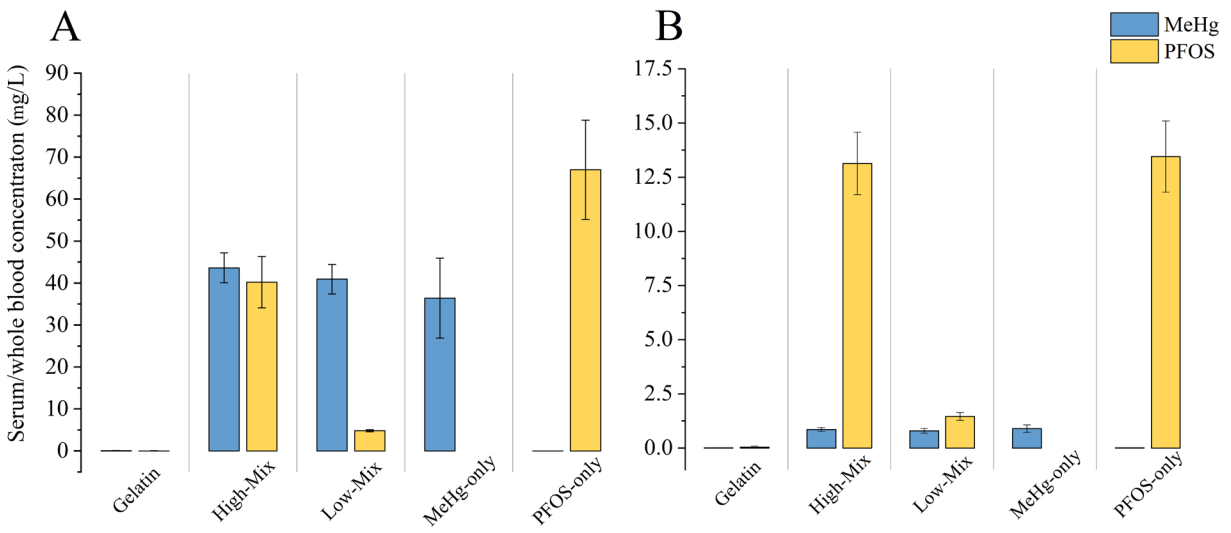

Figure 2-1. PFOS (yellow, ng/mL) in serum and MeHg (blue, µg/L) in whole blood (mean ± SE) of control (n=5) and various treated (each n=4), collected at 3-weeks parturition from A) dams, and B) offspring.

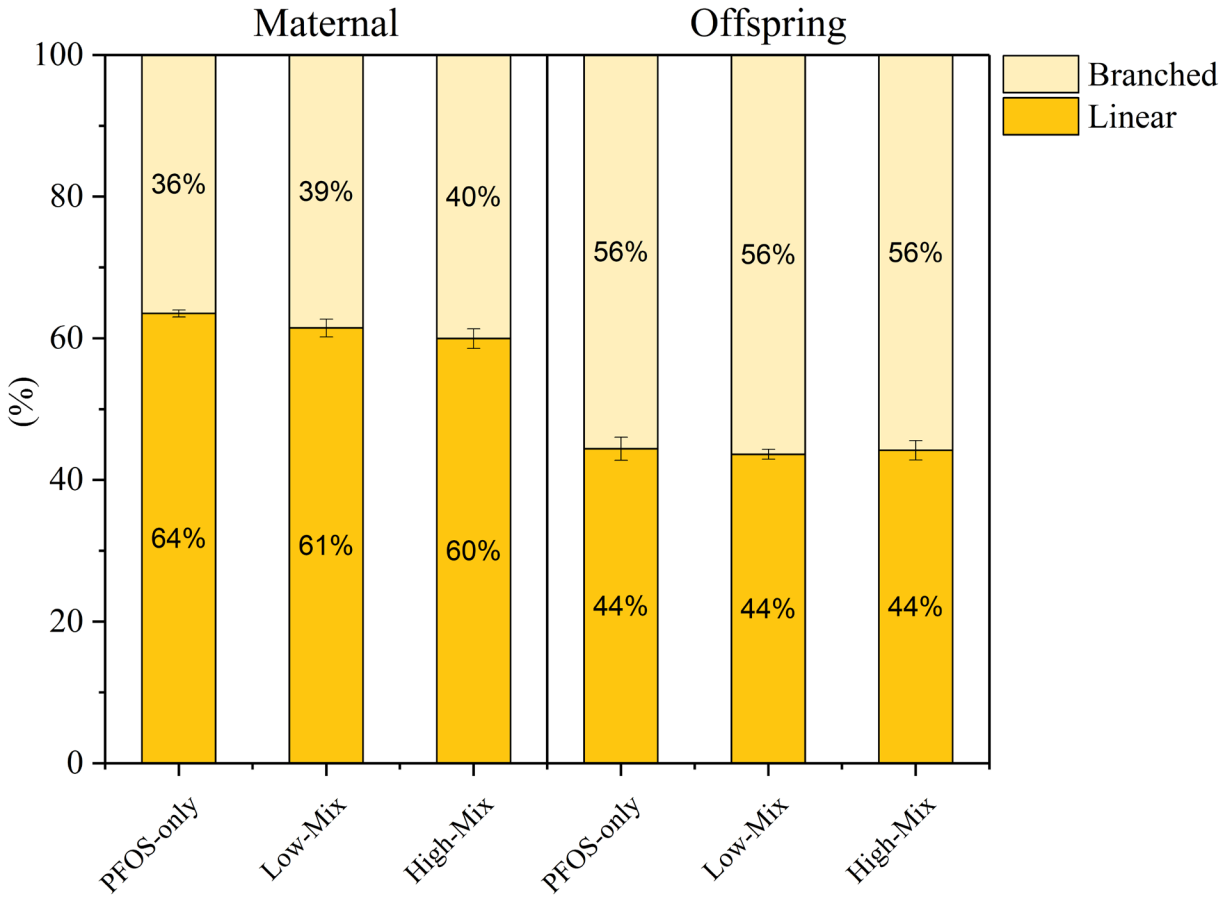

Figure 2-2. Ratio of linear to total branched PFOS isomers in serum (mean ± SE) of A) dams and B) offspring at 3 weeks post-parturition of PFOS-only (1 mg/kg), Low-Mix (0.1 mg/kg) and High-Mix (1 mg/kg) of PFOS with MeHg (1 mg/kg) (n = 4).

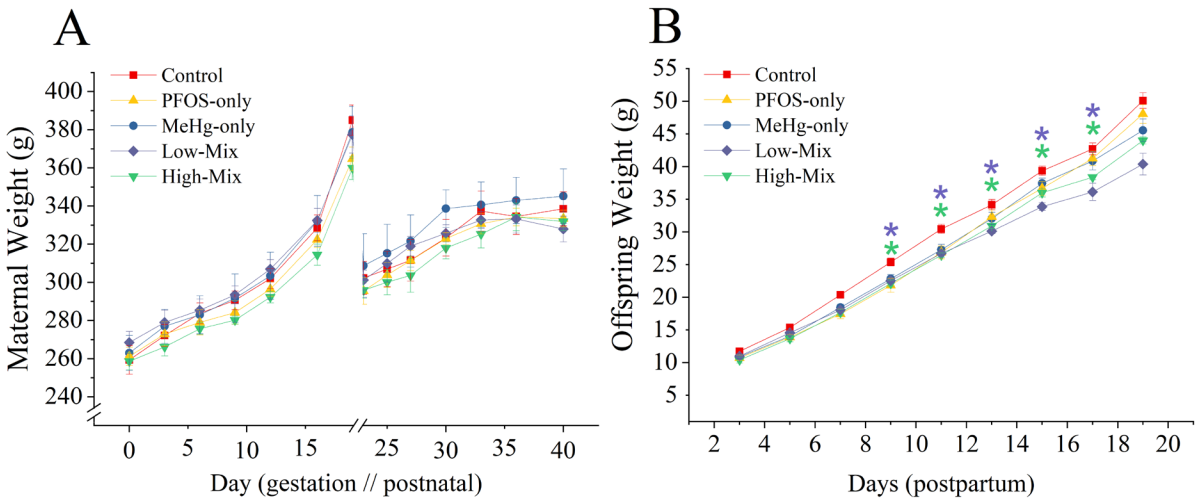

Figure 2-3. Temporal trend for weight gain (mean ± SE) dams from control (red), PFOS-only (yellow), MeHg-only (blue), Low-Mix (purple) and High-mix (green) treatment groups during pregnancy (n = 4-5), and B) offspring during pre-wean stage (n = 16-20).
Dams were monitored continuously; a break indicates the separation of measurements pre- and post-natal
* level of significance from untreated control for treatment group of corresponding colour
$p < 0.05$

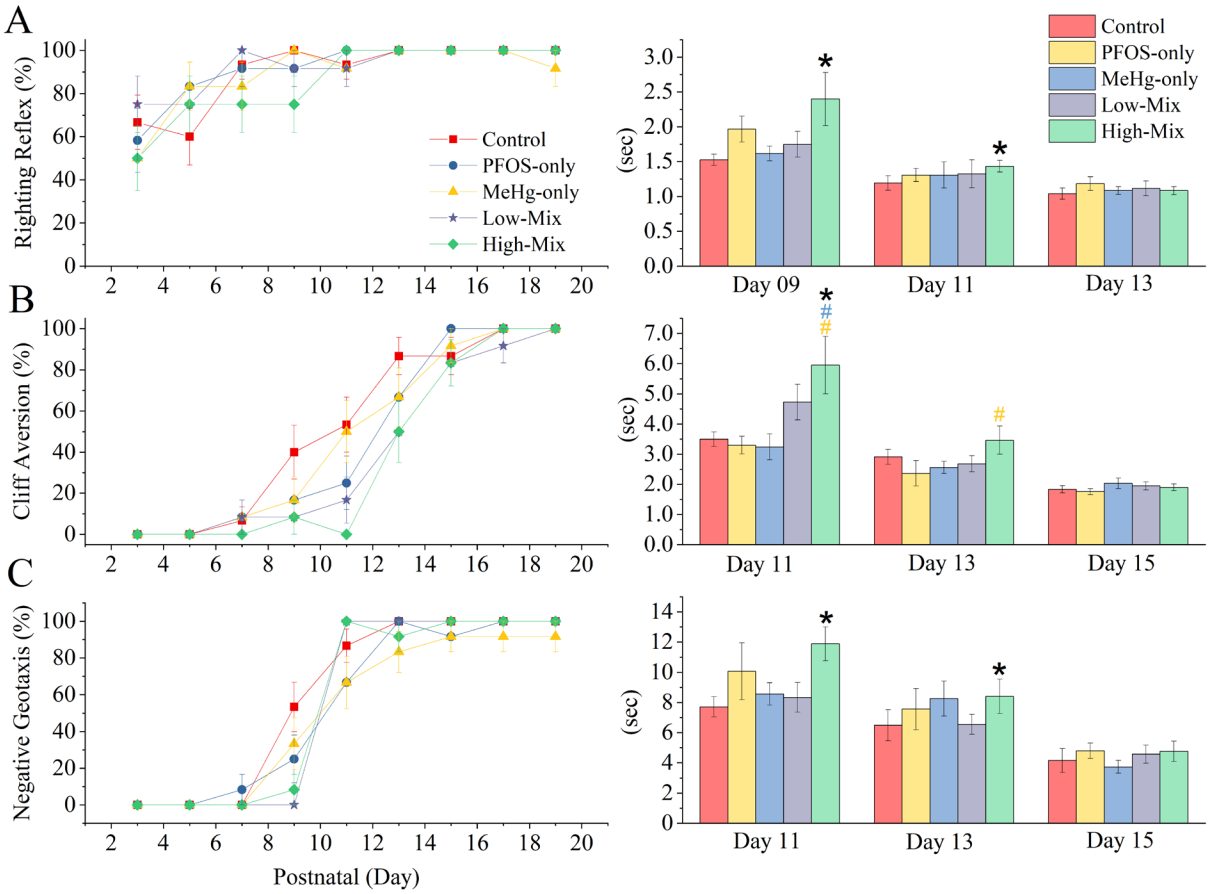

Figure 2-4. Pre-wean growth and performance of newborn offspring (mean ± SE) in tests (A) righting reflex, (B) cliff drop aversion, and C) negative geotaxis from control (red), PFOS-only (yellow), MeHg-only (blue), Low-Mix (purple) and High-mix (green) treatment groups (n = 12 to 15).

The proportion of pups able to complete the task is shown in the left panels, while time taken to complete the task is shown in the right panels

*significant difference from controls ($p < 0.05$)

[#] significant difference from treatment group of corresponding colour ($p < 0.05$)

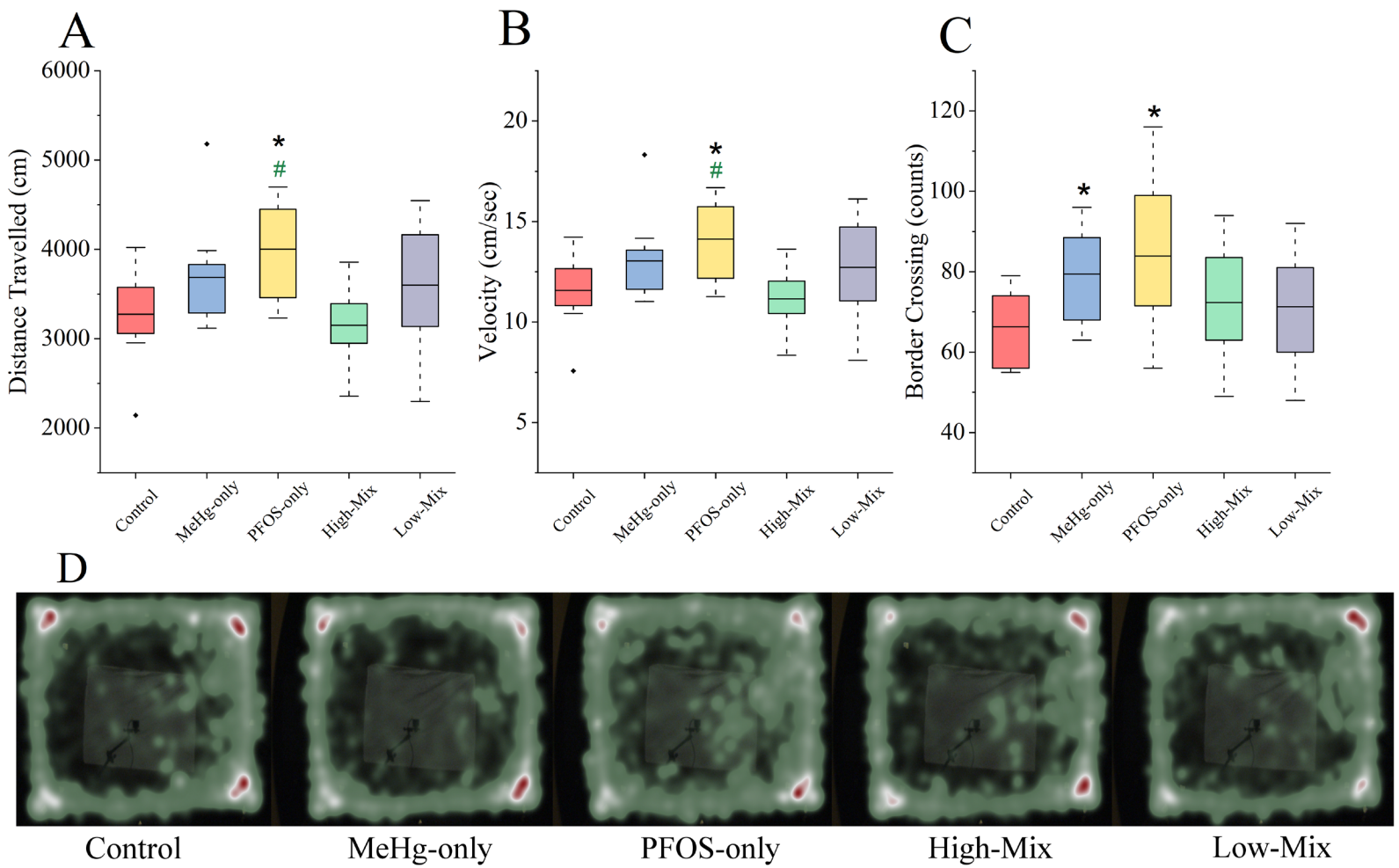

Figure 2-5. Activity of offspring over 5 min exploration in the open field arena from control (red), PFOS-only (yellow), MeHg-only (blue), Low-Mix (purple) and High-mix (green) treatment groups (n=8) of A) distance travelled (cm), B) velocity (cm/s), C) border frequency (#), and D) heatmaps of activity (merged group mean).

*significant difference from controls ($p < 0.05$)

significant difference from treatment group of corresponding colour ($p < 0.05$)

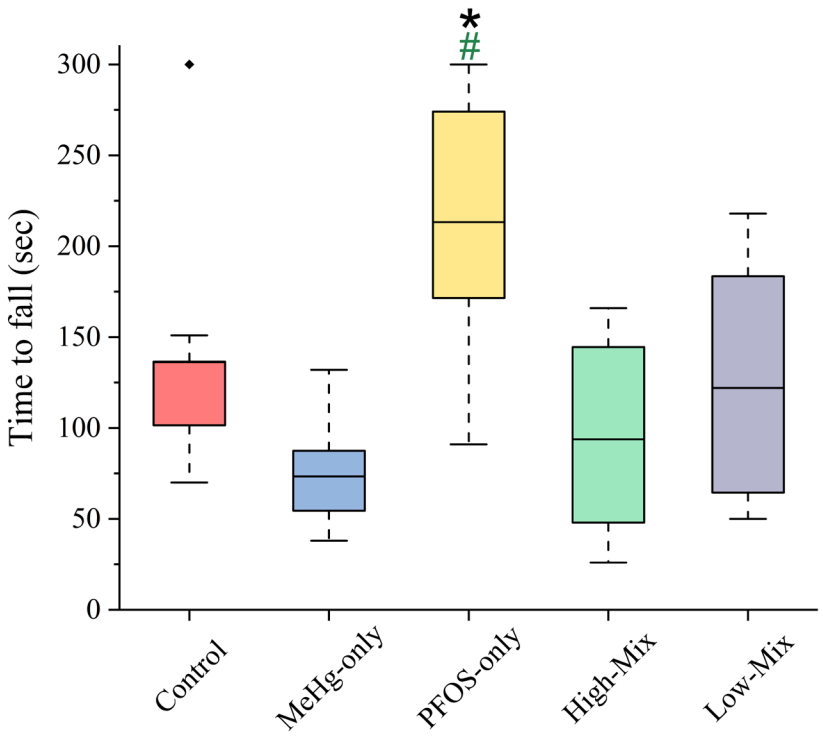

Figure 2-6. Offspring performance (mean ± SE) on the accelerating rotating rod from 5 to 40 rpm over 5 min from control (red), PFOS-only (yellow), MeHg-only (blue), Low-Mix (purple) and High-mix (green) treatment groups (n = 8).

* significant difference from controls ($p < 0.05$)

significant difference from treatment group of corresponding colour ($p < 0.05$)

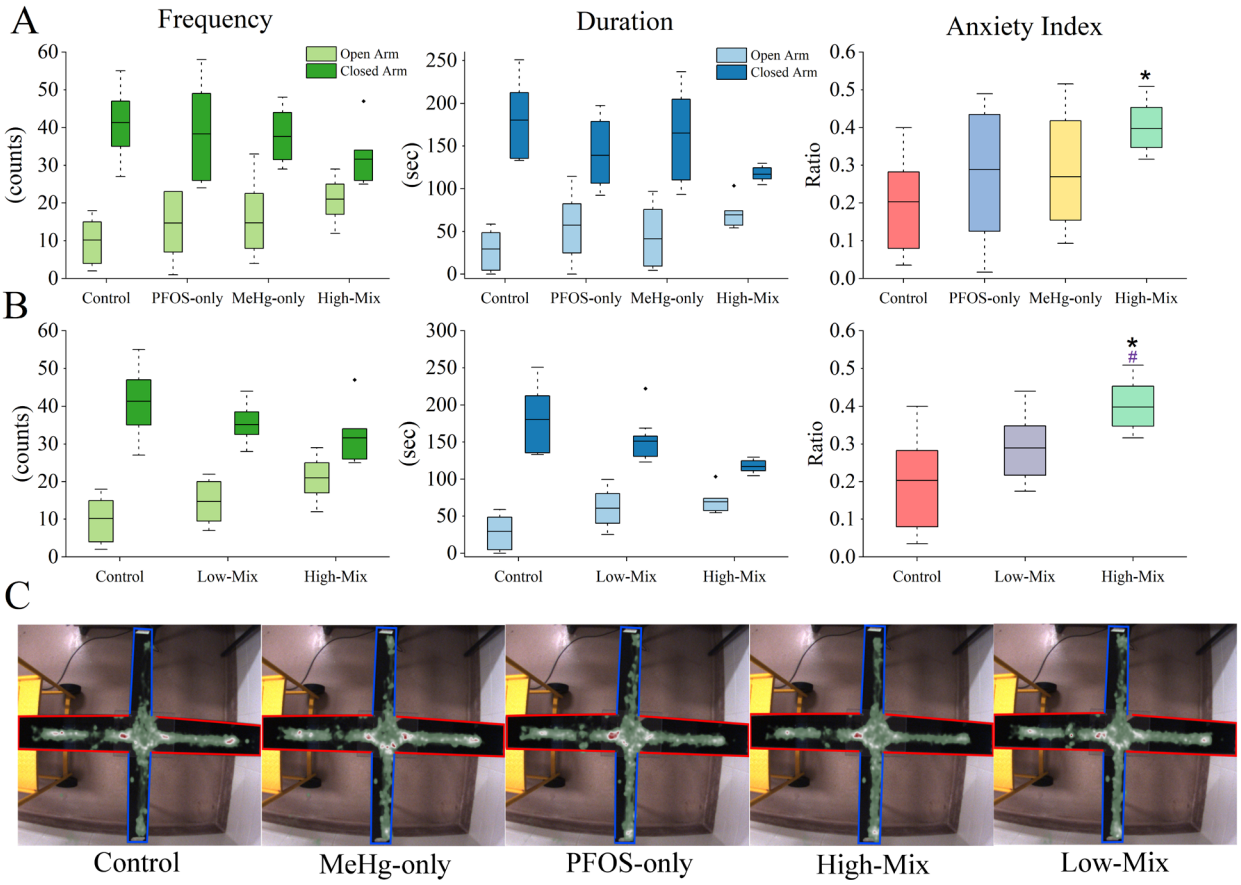

Figure 2-7. Offspring anxiety-related behavioural activity on the elevated plus maze in controls compared to various treatments (n = 8). A) testing hypothesis that combination (MeHg+PFOS) gives different result from PFOS-only or MeHg-only, and B) testing hypothesis that PFOS (Low-Mix or High-Mix) results in a difference at constant MeHg. Data (mean ± SE), includes frequency of arm entries (left panels), arm duration (middle panels), and calculated anxiety index (right panels). C) heatmaps of mean group activity within walled-arm (red outline) and open-arm (blue outline) platforms.

*$p < 0.05$, level of significance from control

$^{\#}p < 0.05$, level of significance from treatment group of corresponding colour

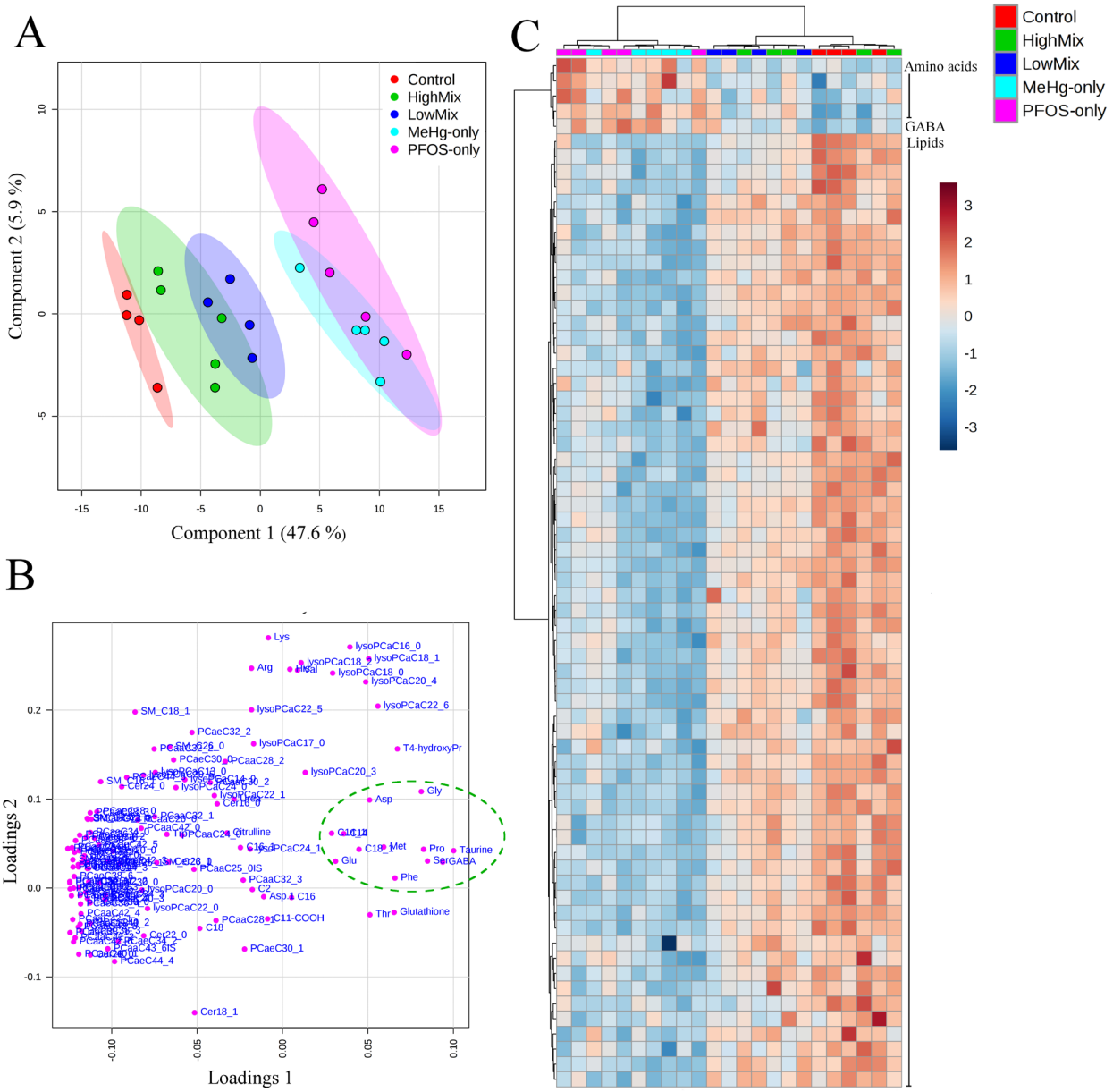

Figure 2-8. A) PLS-DA scores plot, B) loadings plot, and C) hierarchical cluster analysis of significant metabolites contributing to the PLS-DA model quantified in offspring cortex (n=5 per treatment). Metabolites from PFOS-only (purple) and MeHg-only (teal) groups were significantly different from Control (red) and the combined exposure groups Low-Mix (green), and High-Mix (blue).
A colour gradient shows metabolite-specific responses, representing increasing (red), and decreasing (blue) or no change (white) in concentration between treatment groups.

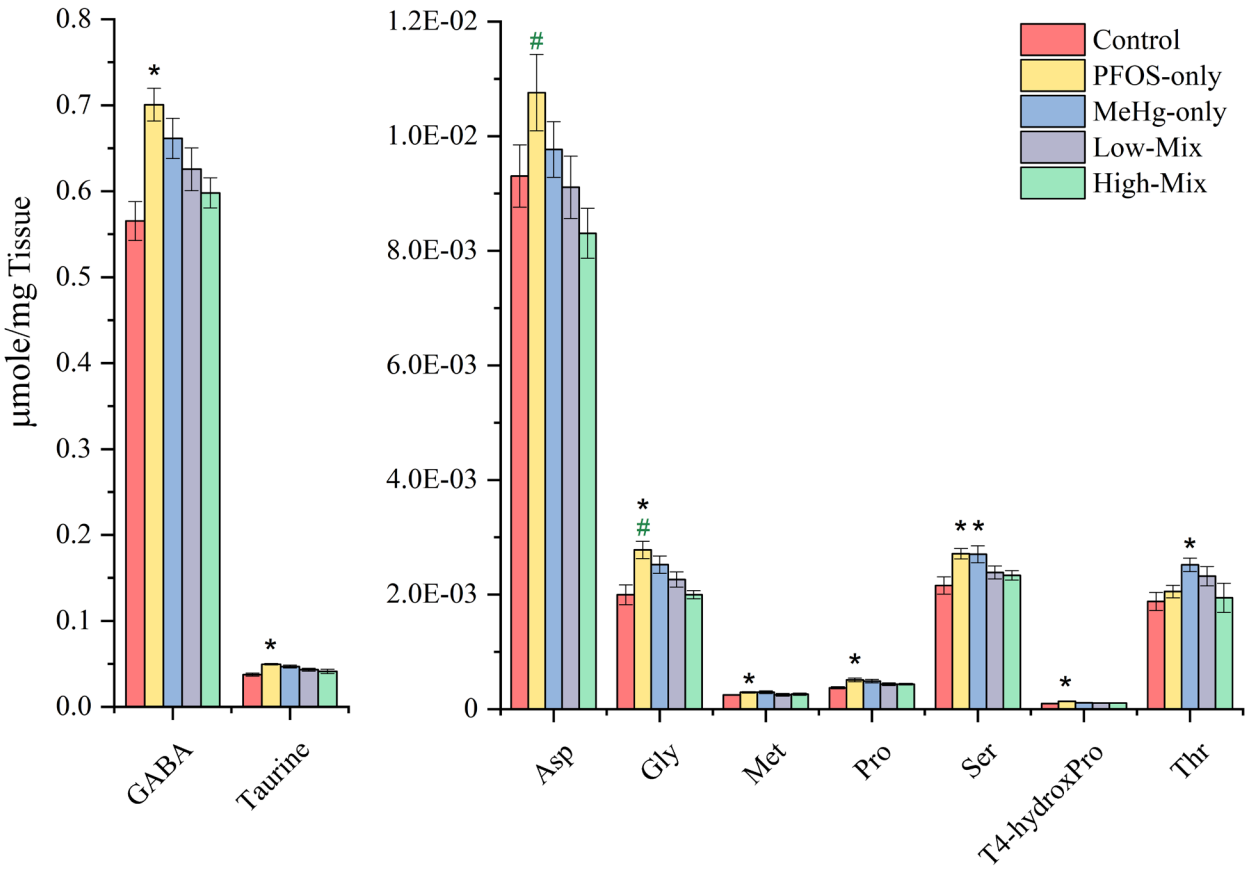

Figure 2-9. Concentration (µmole/mg) of amino acids considered to be significant features in the cortex layer of rat offspring from control (red), PFOS-only (yellow), MeHg-only (blue), Low-Mix (purple) and High-mix (green) treatment groups (n = 5).

*significant difference from controls ($p < 0.05$)

significant difference from treatment group of corresponding colour ($p < 0.05$)

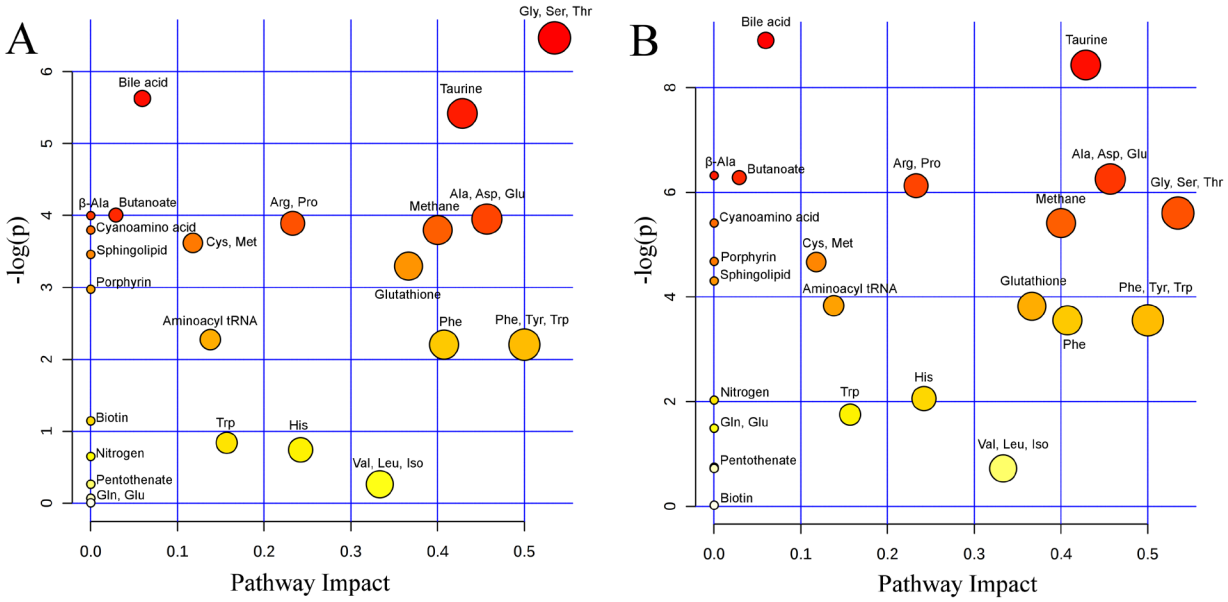

Figure 2-10. Metabolic pathways influenced by altered concentrations of metabolites in (A) MeHg-only and (B) PFOS-only offspring cortex (n = 5). Metabolic pathways are identified as slightly (yellow), moderately (orange) or significantly (red) altered based on level of significance (-log(p), y-axis), as well as the number of metabolites involved (increasing size).

Chapter 3

Longitudinal Analysis Reveals Early-Pregnancy Associations between Perfluoroalkyl Sulfonates and Thyroid Hormone Status in a Canadian Prospective Birth Cohort

3.1 Introduction

Thyroid hormones are important in critical periods of neurodevelopment, including neurogenesis, neuronal migration, proliferation, and myelination[1,2]. Maintenance of an adequate maternal supply of thyroid hormones is, therefore, essential for healthy fetal and postnatal neurodevelopment[3]. In humans, fetal production of thyroxine (T4) and triiodothyronine (T3) is not established until late in the first trimester[4], and until this point the fetus relies on the maternal supply[5]. Thus, early stages of pregnancy exert stress on the hypothalamic–pituitary–thyroid (HPT) axis[6].Throughout pregnancy, thyroid hormone concentrations are dynamic but tightly controlled, and although reference ranges exist for the general population (e.g., 0.45 to 4.5 mU/L for thyroid stimulating hormone (TSH)), there is uncertainty in reference ranges throughout pregnancy. Hormone levels may vary by gestational age, number of fetuses, and between populations[5].

Alterations of maternal thyroid hormone status are linked to adverse birth outcomes and child development[7]. Maternal hypothyroidism, defined by elevated TSH with free (i.e., unbound) T4 (FT4) in the reference range, has been associated with spontaneous abortion, preterm birth, placental abruption, low birth weight[8–10], and lower scores on neuropsychological tests in children[11]. Maternal hypothyroxinemia, defined as FT4 in the lowest 10th percentile without a compensatory increase in TSH, has been associated with lower psychomotor development and delayed mental and motor function in infants[12–14].

Perfluoroalkyl acids (PFAAs) are among the most prominent organic contaminants in human blood, with perfluorohexane sulfonate (PFHxS), perflurooctanoate (PFOA) and perfluorooctane sulfonate (PFOS) present at highest concentrations in Canadian[15] and American[16] populations. Historic and ongoing production of these compounds, combined with their environmental persistence and bioaccumulation potential has led to their global distribution and accumulation in people and wildlife[17]. Dietary intake is a major pathway of PFAA

exposure[18–20], particularly where fish and seafood are major dietary items[21,22], but PFAAs or related precursors may also be present in carpeting, textiles, indoor air and household dust[23–27].

In rodent developmental toxicology studies, PFOS generally elicits effects on thyroid hormone metabolism that are consistent with hypothyroxinemia[28–31]. In monkeys exposed to PFOS[32] or PFOA (i.e. ammonium perfluorooctanoate)[33], subtle alterations in T3 and T4 homeostasis were induced. There have been fewer studies of PFHxS, but one early rat study reported no developmental, nor reproductive toxicity, even at high doses (10 mg PFHxS/kg-d)[34]. More recent studies have reported that PFHxS-exposed pregnant rats and their offspring had reduced serum T4 measured post-pregnancy[35], while in mice, dams had increased liver weight and slight reductions in mean live litter sizes, but no development effects were noted in pups[36].

Epidemiological studies of maternal thyroid disruption by PFAAs have been conducted in Asia, Europe and North America, but with differences in experimental design, including differences in measurement timing for thyroid hormones or PFAAs[37–44]. Positive associations are often reported between certain PFAAs and TSH[37,42–44], suggesting that PFAA exposure may be a risk factor for maternal hypothyroidism. However, as described in a recent review, associations of PFAAs with TSH were not always significant ($p < 0.05$); moreover, reported associations of PFAAs with T3 and T4 have been inconsistent between studies, and future well-designed studies are suggested to confirm the nature of these relationships[45]. Due to the dynamic nature of thyroid hormone concentrations throughout normal pregnancy[6,46], we propose a longitudinal design with repeated measurements of thyroid hormones across the gestational period. A previous study made repeated measurement of thyroid hormones to assess relationship with PFAAs, but this included only one timepoint during the pregnancy, with all other measurements after birth[37].

Additional stressors to the thyroid system may also contribute to variability between studies. In the US general population, PFAAs were associated with changes in thyroid hormone regulation, but only in a subset of participants considered both iodine deficient and had tested positive for thyroid peroxidase antibody (TPOAb)[47], a marker of autoimmune hypothyroidism (i.e. Hashimoto's disease). Similarly, in a Canadian birth cohort, PFAAs (PFNA, PFOA and PFOS) were positively associated with TSH, but only in women with high TPOAb[44].

TPOAb has since been incorporated as a covariate into models of PFAAs and other contaminants to evaluate their effects on maternal thyroid hormone status[48,49], important as a high percentage of pregnant women with hypothyroidism (ranging from 31 to 77 %) have elevated levels of TPOAb[50,51]. In the current study we tested a new hypothesis that co-exposure to mercury (Hg) may confound the effects of PFAAs. Maternal exposure to Hg has deleterious effects on cognition and motor development of offspring, and has been associated with changes in maternal thyroid hormone status, particularly T3[52,53]. Moreover, like PFAAs, dietary intake of fish is a major source of Hg exposure (i.e., methylmercury). Previous epidemiological studies have not considered co-exposure of PFAAs with Hg, but for pregnant rats the combined exposure of PFOA and Hg caused non-additive changes in gene expression of offspring[54]

The aim of the current investigation was to examine the longitudinal association between maternal PFAA exposure and thyroid hormone status in the prospective Canadian pregnancy cohort study known as APrON (Alberta Pregnancy Outcomes and Nutrition). With measurements of thyroid hormone status at three timepoints during pregnancy, and once in the post-partum period, while also considering Hg co-exposure and TPOAb status, this is a large (n = 494) and highly detailed investigation of risk factors for maternal thyroid disruption. This is also the first such study to use an isomer-specific analysis for the PFAAs, which can have different pharmacokinetics and placental transfer[55–57], and isomer-specific associations have already been noted for birth weight and gestational age[58].

3.2 Methods[VI]

3.2.1 Study Participants and Blood Sample Collection[VII]

The study protocols were approved by the University of Calgary Health Research Ethics Board, and the University of Alberta's Human Ethics Research Board. Participants provided

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[VI] I was responsible for experimental design, PFAA method development and subsequent analysis and quantitation in maternal plasma.

[VII] Participant recruitment, data, and sample collection was conducted by members of the APrON team.

written informed consent prior to sample or data collection. Recruitment to the APrON longitudinal Canadian pregnancy cohort was between March 2009 and July 2012. A full description of recruitment methods, rationale for APrON and a detailed description of the cohort is published elsewhere[59,60]. The population for the current study consists of a subset of women from APrON (n = 494) residing in Calgary, recruited prior to 18 weeks gestation (Timepoint 1, mean gestation = 14.3 weeks) and conceived naturally without the use of fertility hormones or assisted reproductive techniques. A detailed description of the sample population and selected covariates is described in Appendix B and listed in Table 3-1. Data were collected through in-person interviews, administration of a first-visit questionnaire, and follow-ups at each timepoint. Details of the assessments have been published[59], but included data collection on diet, physical activity, mental health, medical history, and demographics. For this investigation, potential covariates and confounders included maternal age, education, household income, ethnicity, parity, medical conditions, as well as a history of smoking, alcohol, and recreational drug use.

Plasma or sera were available for most women in each trimester of pregnancy: Timepoint 1 (< 13 weeks gestation, n = 167), Timepoint 2 (14 to 27 weeks gestation, n = 487), and Timepoint 3 (27 to 40 weeks gestation, n = 465). Additional samples were available from 3 months post-partum, Timepoint 4 (n = 479). The majority of PFAA and thyroid analyses were measured in plasma, but serum was utilized when plasma was not available (14 % of all samples). All collection and storage materials were tested for background contamination using HPLC-grade water as a surrogate matrix, and no contamination was detected in any materials. A complete description of blood collection, QA/QC protocols, and analyte recoveries are in Appendix B.

3.2.2 Thyroid Hormone Analysis [VIII]

Free triiodothyronine (FT3), free thyroxine (FT4), thyroid stimulating hormone (TSH), and thyroid peroxidase antibodies (TPOAb) were measured at all time points (Timepoints 1 to 4) in plasma of participants. Chemiluminescent microparticle immunoassay kits were used on the

[VIII] Data from analysis of thyroid hormones was provided by Susan Goruk and Dr. Catherine Field, of the Department of Agricultural, Food and Nutritional Science (University of Alberta)

Architect System (Abbott Diagnostics, Santa Clara, CA). Commercially available controls (Abbott Diagnostics) were included with each run (every 20 samples) to verify uniform precision between runs. In addition to commercial kits, an in-house reference sample (fasting plasma) was also included with each new lot number to ensure instrumental accuracy and consistency of calibration curves with a coefficient of variation < 1 % for thyroid hormones. Six sets of duplicates within each run were included as a QC check with an acceptable coefficient of variation of < 10 %.

3.2.3 Isomer-Specific PFAA Analysis

Extraction of PFAAs was from maternal plasma collected at Timepoint 2 using a modified method from Glynn et al.[61]. Due to their long half-lives[62], PFAA exposure is highly correlated across trimesters[63], making single observations a robust measurement of PFAA exposure. An aliquot of 0.5 mL of plasma was placed in a 15 mL conical polypropylene centrifuge tube containing 1 ng of 8 isotopically labeled internal standards in methanol (MPFAC-MXA, Wellington Laboratories, Guelph, ON) (listed, Appendix B). Plasma extraction was by protein precipitation with 4 mL of acetonitrile (ACN) and sonication at room temperature for 10 min. The plasma/ACN mixture was centrifuged at 2000 rpm for 5 min in an Eppendorf Sorvall ST-40R tabletop centrifuge (Thermo-Fisher Scientific), the supernatant was transferred to a new 15 mL tube, and the pellet was discarded. The supernatant was then evaporated with a stream of nitrogen gas at 40 °C to a volume of 0.3 mL and reconstituted in 50:50 methanol:water to a final volume of 1 mL. This extract underwent dispersive cleanup[64] by transferring to a 1.7 mL Eppendorf tube containing 0.025 g of bulk graphitized carbon (Supelclean ENVI-Carb, Sigma Aldrich), that had been acidified with 50 µL of glacial acetic acid and vortexed for 10 sec. The sample was centrifuged for 10 min at 10,000 rpm (Sorvall Legend Micro 21R, Thermo Scientific) and the top 0.5 mL was transferred to a glass auto-sampler vial.

A rapid, isomer-specific analysis of PFAAs was optimized for use with human plasma from the instrument parameters of a previous method by Benskin et al. used for PFAA analysis of landfill leachate[65]. A total of 25 PFAA analytes, including 16 perfluoroalkyl carboxylates (11 linear and 5 branched isomers) and 9 perfluoroalkyl sulfonates (4 linear and 5 branched isomers) were monitored in all maternal plasma samples at Timepoint 2. Analysis was by HPLC-MS/MS with a UFLC-XR Shimadzu HPLC coupled to an API 5000 triple quadrupole mass spectrometer

(Applied Biosystems Sciex, Concord, ON) operating in negative ion mode with multiple reaction monitoring. A detailed description of instrument parameters, median concentration, and detection limits for all PFAAs are provided in Appendix B. An external solvent-based calibration curve was utilized and each linear and branched PFAA was quantified using the appropriate mass labeled internal standard. A 5 µL sample was injected onto an Ascentis Express F5 PFP analytical column (2.7 µm, 90 ˚A, 10 cm × 2.1 mm, Sigma-Aldrich) equipped with an Ascentis Express F5 PFP guard column (2.7 µm, 5.0 mm x 2.1 mm) at 40 °C. Upstream of the injector, two XTerra C18 columns (5 µm, 30 mm x 2.6 mm each, Waters) were in place to separate instrumental background PFAAs from PFAAs in the sample injected to the analytical column. A binary gradient elution was used, including (A) 5 mM aqueous formic acid and 5 mM ammonium formate, and (B) 100 % methanol at 0.2 mL/min. The elution gradient was initially 10 % B, 60 % B by 3 min, 88 % B by 14 min, and 100 % B by 14.5 min, held until 15 min and returned to initial conditions by 16 min with a further 5 min equilibration.

3.2.4 Total Hg (THg) Analysis[IX]

A 100 µL aliquot of maternal blood cell fraction from Timepoint 2 was diluted with 100 µL of deionized water and diluted with a basic solution containing 25 µg/L of iridium as internal standard, 10 µg/L gold, 0.5 g of EDTA in 1% v/v ammonia hydroxide, 2.5% butanol, and 0.05% v/v Triton X100. The resultant 50-fold dilution was then analyzed for THg (Agilent 8800 ICP-MS/MS). Helium was used as collision gas to remove interferences. Two sources of external quality controls were used (Seronorm™ and Clinchek™) at three levels and injected after every 10 samples. The analytical acceptability range was defined as within 20 % of the reference value according to manufacturer guidelines. Duplicates of each sample were run, and for every 10 samples a sample was randomly selected and spiked with the analytes as an additional QC check. Complete details of quality assurance and quality controls, including instrumental limits of detection and quantitation (LOD, LOQ), and THg recovery are described in Appendix B.

[IX] Mercury analysis was conducted by Dr. Amy MacDonald, and Dr. David Kinniburgh of the Alberta Centre for Toxicology (University of Calgary)

3.2.5 Statistical Analysis[X]

Thyroid hormones were measured over time for the same subject. These observations were not independent, violating the assumption of ordinal regression modeling, thus mixed effect modelling was employed to accommodate the correlation structure within observations of the same subjects. All statistical analyses were performed using R.3.3.2. The effect of each potential covariate was evaluated in the separated mixed effect model in the presence of the main predictor, either PFAAs or THg. Covariates with $p < 0.2$ were nominated to enroll in a multiple regression model. The fixed parameters of the multiple mixed models included the main predictor, the covariates from the first step ($p < 0.2$) and a time variable indicating Timepoints. We also estimated subject-specific trajectories by considering a time variable in the random part of the model. For all mixed effect modeling, three correlation structures (unstructured, autoregressive and compound symmetry) were attempted and the structure with the lowest Akaike information criterion – a method of assessing the quality of a model – was used for final model selection. Multicollinearity between covariates was tested and the goodness of fit for all the models was evaluated exploring the models' residuals. Evaluation in statistical models was restricted to PFAAs that were detected in > 80 % of samples in the overall population, and all thyroid hormones (TSH, FT4, and FT3) were log-transformed to account for skewed distributions.

Within each model, the main effect was determined (β main effect), described as the change in outcome per unit change in predictor (PFAA or THg) when time and all significant covariates were adjusted for. All covariates from the univariate models in the first step are listed in Table 3-1. Maternal age, ethnicity and history of smoking were considered significant and adjusted for in multivariate models of FT3, FT4, and TSH, a diagnosed thyroid condition, and a history of drug and alcohol use were also adjusted for in models of FT4, and FT3 respectively.

In addition to the main effect, the outcome at each Timepoint was also considered to determine the time-dependence of PFAA-thyroid hormone associations. For outcomes at Timepoints during pregnancy (β at Timepoint 1, 2, and 3), the significance ($p < 0.05$) of a time

[X] Statistical analysis was conducted by Dr. Elham Khodayari-Moez and Dr. Irina Dinu of the School of Public Health (University of Alberta)

interaction was considered (e.g., the change in the effect of each PFAA on modeled thyroid hormone from Timepoint 1 to Timepoint 2, Timepoint 2 to Timepoint 3, etc.). For the outcome at Timepoint 4 collected at 3-months postpartum (β at Timepoint 4), the significance of an additional interaction ($p < 0.05$) was considered to account for the change in effect during pregnancy to post-birth. A β-coefficient (change in thyroid hormone per unit change in PFAA), and 95 % confidence intervals were calculated for the main effect and at each Timepoint.

To address the secondary hypothesis of effects from external stressors, the PFAA-thyroid hormone models then considered the significance of additional interaction terms ($p < 0.05$). THg (continuous) and TPOAb (categorical, women considered as TPOAb normal, or TPOAb). A detailed description of treatment of TPOAb is found in Appendix B. All interactions included in the model were considered first-order interactions.

3.3 Results

3.3.1 Population Description

Participants had a mean age of 32 (range, 16-43), the majority were Caucasian (88 %), had completed post-secondary education (99 %), and most (82 %) had annual household incomes above $77,000 CAD (Table 3-1). Most participants were either nulliparous or primiparous (91 %). A proportion (25 %) were self-reported to have a history of smoking, defined as having consumed >100 cigarettes over their lifetime. However, the self-reported proportion of current smokers was < 2 % among pregnant participants. Overall, the described population had similar characteristics to a Canadian birth cohort study (n = 152) in the metropolitan area of Vancouver, Canada, that previously reported on PFAA-thyroid associations[44].

3.3.2 PFAA Concentrations in Maternal Plasma

Certain perfluoroalkyl carboxylates (linear-PFOA, PFNA, and PFDA) and perfluoroalkyl sulfonates (PFHxS, linear-PFOS, and most branched PFOS isomers (*iso*, 5*m*, 3*m*, and 4*m*)) were detected in > 99 % of plasma samples. PFHpA, PFUnA, and PFDoA were detected less frequently, in 66, 89, and 55 % of samples, respectively. Total PFOS had the highest median concentration (4.77 ng/mL), followed in descending order by PFOA (2.11), PFHxS (1.03), PFNA (0.69), PFDA (0.25), PFHpA (0.08), PFUnA (0.06), and PFDoA (0.06) (Table 3-2).

Among the major legacy PFAAs, total PFOS, total PFOA, and PFHxS concentrations were comparable to recent measurements in Canadians[63,66]. Spearman correlations showed that PFOA was strongly correlated with PFNA and with PFDA (rho > 0.77, $p < 0.05$), and moderately correlated with PFHpA, PFUnA, PFDoA, PFHxS and PFOS (rho = 0.32 to 0.49, $p < 0.05$). PFOS and PFHxS were moderately correlated (rho = 0.61, $p < 0.05$), and were not correlated with any other PFAA, except for the moderate correlation with PFOA noted above.

For PFOS isomers of PFOS, linear PFOS was 2.49 ng/mL (69 % of total PFOS) and total branched PFOS was 1.08 ng/mL (31 % of total PFOS) in maternal plasma (Table 3-2). Among PFOS branched isomers, the highest concentration (ng/mL) was for *iso*-PFOS (0.42), followed by 5*m*-PFOS (0.33), ∑3*m*-+4*m*-PFOS (0.23), and 1*m*-PFOS (0.07), that made up 12.7, 9.8, 6.1, and 2.1 % of total PFOS respectively (Table 3-2). All PFOS branched isomers were moderately to strongly correlated (rho = 0.56 to 0.93, $p < 0.05$). Although a broad range of PFOA isomers were also monitored in maternal plasma, only linear-PFOA at a concentration of 2.11 ng/mL (making up 96.8 % of total PFOA) and 0.07 ng/mL of *iso*-PFOA [0.07] (making up 3.2 % of total PFOA) were detectable. However, due to the low frequency of detection of iso-PFOA (< 50 % of total samples), it was excluded from models of associations with thyroid hormones and further mention of PFOA refers only to linear PFOA.

3.3.3 THg Concentrations in Maternal Red Blood Cells

THg was frequently detectable (98 %) in maternal blood cell fraction (Table 3-2). After adjusting for relative volumes of plasma and cell fraction (i.e., Hg concentration in RBC fraction were divided by 2), geometric mean (GM) concentrations (µg/L) of THg in the current study [0.490] were comparable to whole blood concentrations in pregnant women from another recent Canadian cohort (0.491)[67]. Canadian cohort values were lower than recent cohort studies conducted in Norway (1.21)[68] and Korea (3.19)[69], likely due to differences in fish consumption. For example, in Korea[69] and Sweden[70], positive associations have been reported between increased fish intake and Hg. THg concentrations in the current study were lower than in Canadian Cree First Nations (GM = 2.47 µg/L), where higher Hg was also correlated with increased fish consumption[71]. In the current study, THg was not strongly correlated with any PFAA analytes or their isomers, but significant low to moderate correlations were observed between THg and PFDA, and THg and PFUnA (rho = 0.1, and 0.4 respectively, $p < 0.05$).

3.3.4 Thyroid Hormone Concentrations in Maternal Plasma

Concentrations of thyroid hormones (TSH, FT4, and FT3) at all Timepoints are summarized in Table 3-3. TSH increased slightly mid-gestation (Timepoint 2), whereas decreasing trends for FT4 and FT3 were observed throughout the pregnancy (Timepoint 1 to 3). FT4 subsequently increased at 3-months postpartum (Timepoint 4). An increase in TSH between the first and second trimester, and parallel patterns of decreasing FT4 and FT3 have been noted previously, but levels generally remain in the reference range[46].

TPOAb data have been used as a binary variable (i.e., high/normal) in previous investigations of PFAAs and thyroid hormones. Cutoff values used have ranged from low (i.e., 9 mIU/mL)[44,47,52], to moderate (34 to 50 mIU/mL)[37,51] to high (90 mIU/mL)[72]. Here, we used 9 mIU/mL to be consistent with previous PFAA studies[44,47,52], and because this value is the threshold for eliciting disturbance to immunological functioning of thyroid tissue[73]. The range of women with elevated TPOAb in the current study was 5 to 15 % (Table 3-3), which is comparable but slightly wider than the reference range for euthyroid women (i.e., 6 - 12 %) based on previous studies of thyroid hormone status and TPOAb[51,74–76]. Including, and adjusting for hypothyroidism, in models of the current population may have contributed to increased proportion of women with elevated TPOAb. In women categorized as high TPOAb, using a Student t-test, I found that in women categorized as high TPOAb, TSH was significantly higher early in pregnancy (Timepoint 1 and Timepoint 2), and FT4 was significantly higher postpartum (Timepoint 4) compared to normal TPOAb women ($p < 0.05$).

3.3.5 Associations between PFAA or THg Exposure and Maternal Thyroid Hormones

Significant main effect associations ($p < 0.05$) between PFAAs and maternal thyroid hormones (TSH, FT4, FT3) and calculated associations at each timepoint are summarized in table 3-4. All remaining main effect associations of PFAAs with thyroid hormones and interactions are listed in Appendix B.

TSH was positively associated with several perfluoroalkyl sulfonates (PFHxS, ∑Br-PFOS, and 5*m*-PFOS) during pregnancy, but not with any perfluoroalkyl carboxylates, nor THg (Table 3-4). The strength of the significant associations was dependent on time, with all three associations significant in early pregnancy (Timepoint 1); only PFHxS was significant in the

second trimester (Timepoint 2), and none were significant late in pregnancy (Timepoint 3). At 3-months postpartum (Timepoint 4), a significant association with linear-PFOS was revealed, and association with 5*m*-PFOS was significant again, but with a decreased coefficient compared to earlier in gestation (Timepoint 1). For PFHxS, ∑Br-PFOS, and 5*m*-PFOS, the relative trends are similar over time (Fig. 3-1 A-C), with strong dose-dependent positive associations at Timepoint 1, which weaken by Timepoint 2, disappear by Timepoint 3, and reappear post-partum. It is concerning that the most significant and strongest associations occurred in the earliest stage of pregnancy when the fetus may be most susceptible to subclinical maternal hypothyroidism.

For FT4, the only significant association was a negative association with PFHxS at all Timepoints (Table 3-4). This can be visualized over time for increasing inter-quartile concentrations of PFHxS (Fig. 3-2A), demonstrating the consistent dose-dependent association despite the expected decrease in FT4 during the first and second trimester, as well as the subsequent postpartum rise (Table 3-3). These significant associations for PFHxS and FT4 are consistent with the positive associations between PFHxS and TSH at Timepoints 1 and 2, because the function of elevated TSH is to stimulate the thyroid gland to produce more T4 in response to low circulating T4 and T3 (Fig. 1-3).

For FT3, the only significantly associations were for THg and 1*m*-PFOS (Table 3-4). In the case of THg, a significant negative association with FT3 was observed consistently across all Timepoints. This can be observed visually as a dose-response decline in FT3 with increasing median quartile concentration of THg (Fig. 3-2B). Increasing 1*m*-PFOS concentrations were positively associated with FT3 at 3-months postpartum (Timepoint 4), but not at other Timepoints.

The significant associations between TSH and the branched isomers (∑Br-PFOS, 5*m*-PFOS), or between FT3 and 1*m*-PFOS, would likely have been missed in this study without the isomer-specific analysis. This is because the branched isomers are relatively minor, and the major isomer (linear-PFOS, 69% of total PFOS) was not associated with TSH during pregnancy (Table 3-4), nor with FT4 or FT3 at any Timepoint. In fact, by summing all linear and branched isomers we confirmed that total PFOS was not associated with TSH, FT4, or FT3 (Appendix B). A recent review pointed out that previous studies of PFOS and thyroid disruption have been inconsistent[45], and we propose that 'total-PFOS' analytical methods have contributed to this.

Including an interaction term for THg co-exposure did not influence any of the above noted associations for PFAAs. However, one significant new association was revealed, a positive association between ∑3*m*+4*m*-PFOS and TSH ($p < 0.05$) (Table 3-4) when including the interaction of THg co-exposure. This indicates that increasing concentrations of ∑3*m*+4*m*-PFOS are associated with TSH, but that increasing concentrations of THg interact to weaken the association ($\beta = -0.30$, $p = 0.047$). This new relationship agrees with the general association for ∑Br-PFOS and 5*m*-PFOS which were significantly positively associated with TSH in early pregnancy (Timepoint 1) and at 3-months postpartum (Timepoint 4) (Table 3-4).

3.3.6 Inclusion of TPOAb status

Including a TPOAb interaction term into the model, whereby participants were classified as either normal (< 9 IU/mL) or high (> 9 IU/mL) at each time point, did not significantly alter any of the observed main effect associations (Table 3-4). Thus, significant associations with thyroid hormones for PFHxS, ∑Br-PFOS, 5*m*-PFOS and THg are true in the entire sample population, not only in the high TPOAb group.

Nevertheless, new significant associations were revealed after inclusion of TPOAb status (Table 3-5). PFUnA was positively associated with FT4 in the high TPOAb group only. The association was consistent at all measured timepoints; thus, the result is unlikely spurious because it was consistent throughout pregnancy and at 3-months postpartum. The branched PFOS isomer, 1*m*-PFOS, was also associated with TSH when including TPOAb status, but the result was time-dependent (Table 3-5). Specifically, in the normal TPOAb group, there was a significant positive association between 1*m*-PFOS and TSH at Timepoints 1 and 2, but not at Timepoints 3 and 4. The positive association of 1*m*-PFOS with TSH is not surprising, as the normal TPOAb group represents 85% of the population, and similar longitudinal trends were observed for the sum of all PFOS branched isomers (∑Br-PFOS), as well as 5*m*-PFOS. More unexpected was the opposite association in the high TPOAb group, whereby 1*m*-PFOS was inversely associated with TSH (Table 3-5). This association was not significant early in gestation (Timepoints 1 and 2) but became stronger over subsequent timepoints, with significant negative associations in late pregnancy and at 3-months postpartum (Timepoints 3 and 4). This vulnerable TPOAb subgroup may be more susceptible to the effects of environmental contaminants such as

PFAAs, and these results suggest a unique response compared to the larger normal TPOAb population.

3.4 Discussion

Maternal thyroid hormone homeostasis is under inherent stress during pregnancy. Increasing estrogen in early gestation coincides with increased T4-binding globulin, resulting in increased total T3 and T4[6] which continues throughout pregnancy to meet maternal and fetal demand, even after onset of fetal thyroid function at 16-20 weeks[77]. Elevated TPOAb, which occurs in 6 – 12 % of pregnant women, is considered a clinical marker of autoimmune thyroiditis (Hashimoto's disease) and indicates additional stress on the thyroid that increases the risk of adverse birth outcomes[51,78]. Thus, pregnant women may be vulnerable to thyroid disruption from the additional stress of environmental contaminants that act as endocrine disruptors, such as PFAAs and Hg.

Epidemiological studies of pregnant women are complicated by gradual changes in maternal physiology, and by changing concentration of thyroid hormones throughout the pregnancy[79–82], also observed here (Table 3-3). Given the susceptibility of pregnant women to thyroid disruption, while also considering the biological importance of understanding the timing of any disruption, longitudinal studies are particularly well suited to studies of maternal thyroid disruption.

In prospective cohorts, longitudinal approaches have several advantages over other study designs, including identification of the timing, trends, or recurrence of adverse outcomes[83]. This study on PFAAs is the first to measure thyroid outcomes at multiple time points during the gestational period, and important time-dependent associations were revealed for the first time (Table 3-4). Statistical models using repeat measures accounted for changes in thyroid hormone levels over time, revealing significant relationships between PFHxS and TSH, or PFOS isomers and TSH that were strongest early in pregnancy, gradually disappeared by the third trimester, and in some cases, reappeared post-partum. These trends suggest a window of vulnerability in early pregnancy at a time when the fetus is almost entirely dependent on adequate maternal supply of thyroid hormones. In other cases, the longitudinal design revealed no trend over time but demonstrated consistent significant associations at each time point. For example, the negative

associations between PFHxS and FT4, and between THg and FT3 (Table 3-4). Such consistent results provide great internal validity that the associations are not spurious.

In the current study, overall results for PFHxS were particularly strong and compelling. Higher maternal PFHxS exposure was positively associated with TSH (time-dependent) and negatively associated with maternal FT4 (not time-dependent). This is consistent with previous findings of a positive association of PFHxS with TSH[42–44] and negative association with FT4I, an index of free T4 in circulation[49]. Taking this into consideration with the current dataset showing a consistent dose-response between increasing PFHxS concentrations and declining FT4 at all Timepoints, evidence is building for a causal relationship[84]. Several mechanisms have been proposed to explain the thyroid disrupting effects of PFAAs, including increased hepatic clearance and excretion of T4 through glucuronidation[85], deiodinase enzyme-mediated conversion of T4 to T3[85], and the competitive displacement of T4 from thyroid hormone binding proteins in serum[86,87]. In fact, compared to other PFAAs, PFHxS has the highest in vitro competitive binding potency to human transthyretin, an important human T4 serum transport protein[86]. Our findings are in support of this latter mechanism, as it has been proposed that competitive displacement of T4 by PFAAs leads to a transient increase in FT4, but that the resulting clearance from induction of T4 metabolism ultimately reduces FT4 at steady-state[40]. Moreover, dose-dependent reductions in plasma FT4 have also been observed in chicken embryos exposed to PFHxS[88]. Notwithstanding such in vitro results, this mechanism required relatively high concentrations of PFAAs, and the validity in vivo at lower concentrations is questionable[89]. Nevertheless, observed associations in this study and previous studies are consistent with this mechanism.

According to NHANES data between 1999-2000[90] and 2007-2008[47], human exposure to PFOS drastically declined (geometric mean declined from 30.4 to 13.5 ng/mL) whereas PFHxS exposure was effectively unchanged (2.1 and 1.9 ng/mL, respectively) over the same time period. More recent monitoring of PFAAs in plasma samples collected from American Red Cross donors in 2015 found further decreased PFOS (4.3 ng/mL), and to a lesser extent, PFHxS (0.9 ng/mL)[16], which was at comparable concentrations to the current study (Table 3-2). Human PFOS exposure has declined much faster than PFHxS, and PFHxS may soon be the most prominent PFAA in humans if it is not targeted for mitigation.

To my knowledge, this is the first epidemiological study to include an isomer-specific analysis of PFAAs for investigation of maternal thyroid hormone disruption. This is a limitation of previous work because PFOS and PFOA are present in human blood as various linear or branched isomers that are known to have different pharmacokinetics[55,56]. Isomer-specific analysis is particularly important for PFOS which was historically manufactured as a mixture of linear (70 % of total PFOS) and branched (30 %) isomers. Populations around the world have wide-ranging proportions of branched PFOS isomers; for example the branched PFOS content in samples from China (52 %)[91] were higher than in Norway (30-47 %)[20,92] or Vietnam (17 %)[93]. Higher proportions of branched PFOS isomers have been detected in fetal cord blood (24 %, and 36-54 %) compared to paired maternal samples (17 %, and 27-44 %)[57,94] and branched PFOS isomers have higher transplacental transfer efficiencies than linear PFOS[95].Thus, the associations between branched PFOS isomers and maternal thyroid disruption could also be relevant to thyroid disruption in the developing fetus.

Interestingly, the strongest associations with TSH were for PFHxS and branched PFOS isomers, with no corresponding association for linear PFOS (Table 3-4). PFHxS and branched PFOS isomers may share optimal physical properties or molecular sizes that allow them to interact most strongly with receptor biomolecules that control thyroid hormone homeostasis. Compared to linear PFOS, PFHxS and branched PFOS molecules are shorter in length and less hydrophobic, for example eluting earlier than linear PFOS in reversed phase chromatography. PFHxS has already been demonstrated to be more optimal than PFOS for displacing T4 from transthyretin[86], but the same studies have yet to be done for branched PFOS isomers. For all these reasons, future epidemiological and toxicological studies of PFOS should consider isomer-specific analysis and isomer-specific toxicity.

The “multiple-hit hypothesis” proposed by Webster et al. states that populations with pre-existing thyroid related conditions may be more susceptible to the effects of thyroid disrupting environmental contaminants (Webster, 2014), supported by evidence of PFAA-induced thyroid dysregulation in a subset of people with high TPOAb and an iodine deficiency[47]. Within the small subgroup of women with elevated TPOAb in the current study, PFUnA was negatively associated with FT4, and 1*m*-PFOS was negatively associated with TSH (Table 3-5). PFOS has previously been reported to have both positive[44] and negative[49] associations with TSH in high TPOAb status pregnant women. Although the mechanism of interaction between PFAAs, thyroid

hormones, and TPOAb is not well understood, these results further support the multiple-hit hypothesis[44].

THg analysis was included in the current study to control for a hypothesized confounding variable, but also to consider any relevant mixture effects on thyroid disruption. In the current Canadian cohort, blood THg was not strongly correlated with serum PFAAs and was not considered a confounder but should be included in future studies where fish intake is higher (i.e., in populations with increased THg exposure). Nevertheless, THg was negatively associated with free T3, similar to previous associations between Hg and total T3 (FT3 was not analyzed) in pregnant women from Quebec, Canada[96], and Spain[97]. In Slovakia, Hg in cord blood was inversely associated with total T3 and FT3 in 6-month old infants[53]. These findings are consistent with the proposed mechanism of action for Hg through enzymatic inhibition of deiodinase activity (enzyme D3, in particular), resulting in reduced T3 production[98,99]. A possible mixture effect between THg and ∑3*m*-+4*m*-PFOS was observed in models of TSH, whereby the main effect of ∑3*m*-+4*m*-PFOS was positively associated with TSH when including THg co-exposure; however, the ∑3*m*-+4*m*-PFOS-THg interaction term was negative, indicating that at higher Hg concentrations association of these PFOS isomers with THg became weaker. Thus, it is possible that THg increases sensitivity to thyroid disruption by PFOS branched isomers but is dependent on the concentration of THg.

Although this was among the largest and most detailed studies of PFAAs and thyroid disruption, I acknowledge some study limitations. Participants all resided in the metropolitan area of Calgary, Canada, and were from a narrow demographic (e.g., high socioeconomic status) that may not be representative of the overall population. This may limit the external validity of our findings when considering other global populations, but it does not decrease the internal validity. Nevertheless, with respect to PFAA exposure, the concentrations of PFAAs in maternal plasma in this study were almost identical to another Canadian birth cohort[44], and similar to other cohorts in Korea[40], China[100], Taiwan[43], and Norway[48]. With respect to iodine sufficiency, this was determined using questionnaires, assuming iodine sufficiency if taking prenatal supplements that included iodine. In the general US population, thyroid hormone status was particularly susceptible to PFAAs for a subgroup with combined high TPOAb status and an iodine deficiency[47]. Thus, future studies might consider inclusion of urinary iodine. Finally, thyroid hormones were measured by immunoassay. Although this is one of the most commonly

employed methods of quantifying thyroid hormones in human samples, radioimmunoassays, such as the one used in this study, are sensitive to serum binding protein concentration, not designed to be used under conditions when protein levels are subject to change (i.e., during pregnancy)[101]. Similar problems resulted in reporting bias and underreporting of FT4 values in animal models[102], but have been deemed less problematic in human samples. Nonetheless, pregnancy cohort studies might benefit from the enhanced specificity and precision of liquid chromatography tandem mass spectrometry (LC-MS/MS) over immunoassays.

3.5 Conclusions

The prospective APrON birth cohort study allowed one of the largest and most detailed studies of maternal thyroid hormone disruption by PFAAs. With three sampling times throughout pregnancy and another sampling time after birth, the longitudinal analysis revealed new trends that are of biological significance, and which could explain variability among previous studies. By considering additional thyroid stressors, THg and TPOAb status, the study also revealed interactions of relevance to the multiple hit hypothesis, and to toxicology of environmental chemical mixtures in human blood, respectively. By using an isomer-specific method for PFAA analysis, the distinct toxicological behavior and relevance of branched PFOS isomers was revealed, which highlights the importance of isomer-specific PFAA methodologies in future epidemiology and toxicology studies. Building on previous literature, there is now very strong evidence in support of causal relationships between PFHxS and PFOS exposure and disruption of maternal thyroid homeostasis in early pregnancy. This information should be considered in future international decisions under the Stockholm Convention, where PFHxS has been nominated for inclusion as a Persistent Organic Pollutant[103], and PFOS is already included but with many current exemptions under its listing in Annex B.

3.6 References

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Table 3-1. List of covariates evaluated in univariate models from participants of the APrON cohort, considered for inclusion in multivariate models

	Type	Category (n)	Median	Range
Maternal age	Continuous	-	32	16 - 43
Education	Categorical	< high school (4) High school (29) Trade/technical school (85) University (233) Post-grad (129) Missing data (16)	-	-
Ethnicity	Categorical	Caucasian (426) Chinese (17) Filipino (5) Japanese (2) Korean (0) Latin American (13) Native/Aboriginal (1) South Asian (7) South East Asian (4) Arab (2) West Asian (0) Black (2) Other (7) Missing data (14)	-	-
Household Income	Categorical	< 20K (11) 20K to 39,999 (21) 40K to 69,999 (53) 70K to 99,999 (107) 100K and above (289) Missing data (13)	-	-
Parity	Categorical	no children (278) 1 previous child (162) 2 previous children (41) 3 previous children (5) 4 previous children (0) Missing data (13)	-	-
Smoking – history prior to pregnancy	Categorical	Yes (372) No (114) Missing (13)	-	-
Smoking during pregnancy	Categorical	Yes (8) No (479) Missing (13)	-	-
Alcohol use during pregnancy	Categorical	Yes (24) No (446) Missing (26)	-	-
Recreational drug use - history	Categorical	Yes (7) No (464) Missing (26)	-	-
Medical conditions	Categorical	Yes (8) No (459) Missing (13)	-	-

Table 3-2. Concentrations, and detection frequency of PFAAs (ng/mL), THg (µg/L), and isomers of PFOS in maternal plasma (n = 494), including isomer proportions of total PFOS (%)

	AM	**GM (SD)**	**Min**	**Median**	**Max**	**% > DL**	
PFAAs							
PFHpA	0.14	0.04 (0.33)	< LOD[A]	0.08	3.87	66.6	
PFOA	2.88	2.12 (3.38)	0.265	2.11	43.3	100	
PFNA	1.05	0.76 (1.27)	< LOD[A]	0.69	15.8	99.0	
PFDA	0.38	0.26 (0.51)	< LOD[A]	0.25	7.01	99.8	
PFUnA	0.21	0.16 (0.15)	< LOD[A]	0.06	1.26	88.5	
PFDoA	0.05	0.03 (0.050)	< LOD[A]	0.06	0.68	55.5	
PFHxS	1.52	1.01 (1.76)	0.03	1.03	15.9	100	
PFOS	5.18	4.54 (2.69)	0.66	4.77	17.9	100	
THg	1.47	0.98 (1.32)	0.03	1.06	8.68	97.6	
Isomers of PFOS							Total PFOS (%)
L-PFOS	2.72	2.34 (1.49)	0.14	2.49	9.15	100	69.0
∑Br-PFOS	1.22	1.08 (0.66)	0.25	1.08	4.77	100	31.0
1*m*-PFOS	0.08	0.06 (0.05)	< LOD[A]	0.06	0.43	96.8	2.1
∑3*m*+4*m*-PFOS	0.26	0.23 (0.14)	0.05	0.23	1.19	100	6.1
5*m*-PFOS	0.38	0.32 (0.23)	0.06	0.33	1.63	100	9.8
Iso-PFOS	0.50	0.43 (0.28)	0.11	0.42	1.92	99.6	12.7

Abbreviations: AM (arithmetic mean), GM (geometric mean), SD (standard deviation), DL (detection limit)
Note: Samples from participants collected at Timepoints 1 (0.6 %), 2 (96 %), 3 (3 %), and 4 (0.4 %)
[A] Value below methods quantitation limits (MQL; ng/mL), detailed in supplementary material

Table 3-3. Thyroid hormone concentrations in maternal plasma over the 3 trimesters of pregnancy (Timepoints 1 to 3) and three months postpartum (Timepoint 4)

		Timepoint 1 (n = 167)		**Timepoint 2 (n = 487)**		**Timepoint 3 (n = 465)**		**Timepoint 4 (n = 479)**	
		AM (SD)	Median (range)	AM (SD)	Median (range)	AM (SD)	Median (range)	AM (SD)	Median (range)
TSH	All	1.48 (1.39)	1.18 (0.15, 3.21)	1.59 (0.87)	1.38 (0.57, 3.15)	1.36 (0.62)	1.28 (0.52, 2.45)	1.25 (1.67)	1.14 (0.07, 2.28)
(mIU/L)	High TPOAb	*3.14 (2.75)	*2.07 (0.48, 8.01)	*2.16 (1.25)	*2.01 (0.59, 4.58)	1.47 (0.86)	1.42 (0.20, 2.57)	1.24 (2.95)	0.53 (0.07, 2.28)
	Normal TPOAb	1.21 (0.71)	1.10 (0.15, 2.52)	1.50 (0.76)	1.33 (0.57, 2.76)	1.35 (0.57)	1.27 (0.54, 2.38)	1.26 (0.55)	1.17 (0.50, 2.28)
FT4	All	15.3 (3.28)	14.9 (11.8, 19.6)	14.3 (2.43)	14.0 (11.6. 17.5)	13.2 (4.04)	12.6 (10.0, 16.6)	14.5 (2.87)	14.03 (11.6, 19.4)
(pmol/L)	High TPOAb	14.1 (4.16)	14.2 (8.9, 19.6)	14.9 (2.75)	14.4 (11.3, 19.6)	14.4 (5.56)	13.4 (10.6, 18.2)	*17.1 (5.61)	*15.8 (11.6, 28.2)
	Normal TPOAb	15.5 (3.06)	15.1 (12.1, 18.7)	14.2 (2.37)	14.0 (11.6, 17.0)	13.0 (3.73)	12.5 (10.0, 15.9)	14.1 (1.91)	13.9 (11.7, 17.0)
FT3	All	4.66 (0.85)	4.62 (3.72, 5.58)	4.58 (0.65)	4.53 (3.69, 5.51)	4.58 (2.14)	4.36 (3.62, 5.41)	4.22 (0.87)	4.13 (3.30, 5.30)
(pmol/L)	High TPOAb	4.20 (0.99)	4.38 (3.46, 4.99)	4.58 (0.88)	4.55 (3.61, 5.47)	4.47 (0.61)	4.47 (3.79, 5.11)	4.79 (1.79)	4.49 (3.16, 7.77)
	Normal TPOAb	4.74 (0.80)	4.69 (3.81, 5.58)	4.58 (0.61)	4.53 (3.70, 5.51)	4.59 (2.29)	4.35 (3.61, 5.42)	4.14 (0.58)	4.10 (3.30, 5.05)
TPOAb[A]		8 (5 %)		73 (15 %)		70 (15 %)		67 (14 %)	

Sample collection: Timepoint 1 (< 13 weeks), Timepoint 2 (14 – 26 weeks), Timepoint 3 (27 – 40 weeks), Timepoint 4 (3 months postpartum). Range was defined as the 5th percentile (lower) and 95th percentile (upper) of the sample population.
* $p < 0.05$, level of significance (high vs. normal TPOAb)
[A] Threshold indicating participants with high TPOAb, n (%) > 9 Iu/mL

Table 3-4. Mixed effects model of overall associations between exposure to PFAAs, THg or PFAA and THg co-exposure with maternal thyroid hormones

	PFAA (interaction)	**Main Effect** β (95 % CI)	**Timepoint 1** β (95 % CI)	**Timepoint 2** β (95 % CI)	**Timepoint 3** β (95 % CI)	**Timepoint 4** β (95 % CI)
TSH	PFHxS [a,b,c]	0.144** (0.139, 0.149)	0.093** (0.025, 0.160)	0.042* (0.007, 0.078)	-0.009 (-0.045, 0.028)	0.049 (-0.001, 0.098)
	∑ Br-PFOS [a,b,c]	0.286* (0.273, 0.299)	0.180* (0.007, 0.352)	0.073 (-0.021, 0.167)	-0.034 (-0.132, 0.065)	0.122 (-0.011, 0.255)
	L-PFOS [a,c]	0.005 (0.096, 0.108)	-	-	-	0.062* (0.007, 0.118)
	5*m*-PFOS [a,b,c]	0.851* (0.815, 0.887)	0.541* (0.055, 1.026)	0.230 (-0.035, 0.496)	0.080 (-0.357, 0.196)	0.411* (0.034, 0.787)
	∑3m+4m PFOS(*THg) [a,d,e]	1.357* (0.060, 2.653)	0.998* (0.106, 1,890)	0.639* (0.036,1.243)	0.281 (-0.343,0.905)	0.9203* (0.173, 1.668)
FT4	PFHxS	-0.006* (-0.012, -0.001)	-	-	-	-
	1*m*-PFOS [a,c]	-0.122 (-0.130, -0.113)	-	-	-	0.333* (0.082, 0.583)
FT3	THg	-0.016* (-0.023, -0.008)	-	-	-	-

Significance level *p < 0.05, **p < 0.01,

Model estimates were calculated using log-values of thyroid hormone concentrations

[a] Effect of PFAAs on thyroid hormones after adjusted for moderately significant covariates (p < 0.2) in univariate models

[b] Association includes time as a significant interaction (all Timepoints)

[c] Association includes birth as a significant interaction (Timepoint 4 only)

[d] Association includes THg as a significant interaction

[e] Interaction of THg, effect sizes within the table were calculated when THg set to a reference value

- value unchanged from main effect, time or birth interactions not significant (p > 0.05)

Table 3-5. Mixed effects model coefficients for associations between PFAAs and thyroid hormones in adjusted models after including interaction term for TPOAb status

		Time	Normal TPOAb	High TPOAb	TPOAb Interaction[c]
			β (95 % CI)	β (95 % CI)	(p-value)
FT4	PFUnA[A]	all	-0.011 (-0.087, 0.066)	-0.240 (-0.456, -0.025)*	0.049
TSH	1*m*-PFOS[A,B]	1	1.970 (0.198, 3.741)*	-0.819 (-3.13, 1.49)	0.008
		2	1.362 (0.218, 2.505)*	-1.427 (-3.32, 0.468)	
		3	0.754 (-0.273, 1.78)	-2.035 (-3.88, -0.190)*	
		4	0.146 (-1.40, 1.69)	-2.643 (-4.83, -0.453)*	

[A] Effect of PFAAs on thyroid hormones after adjusting for significant covariates in univariate models
[B] Significant interaction with time, coefficient calculated for main effect (all) and for each Timepoint (#)
[C] TPOAb interaction, identified level of significance of the difference between normal vs. high TPOAb using a threshold of 9 IU/mL
*p < 0.05, **p < 0.01, level of significance

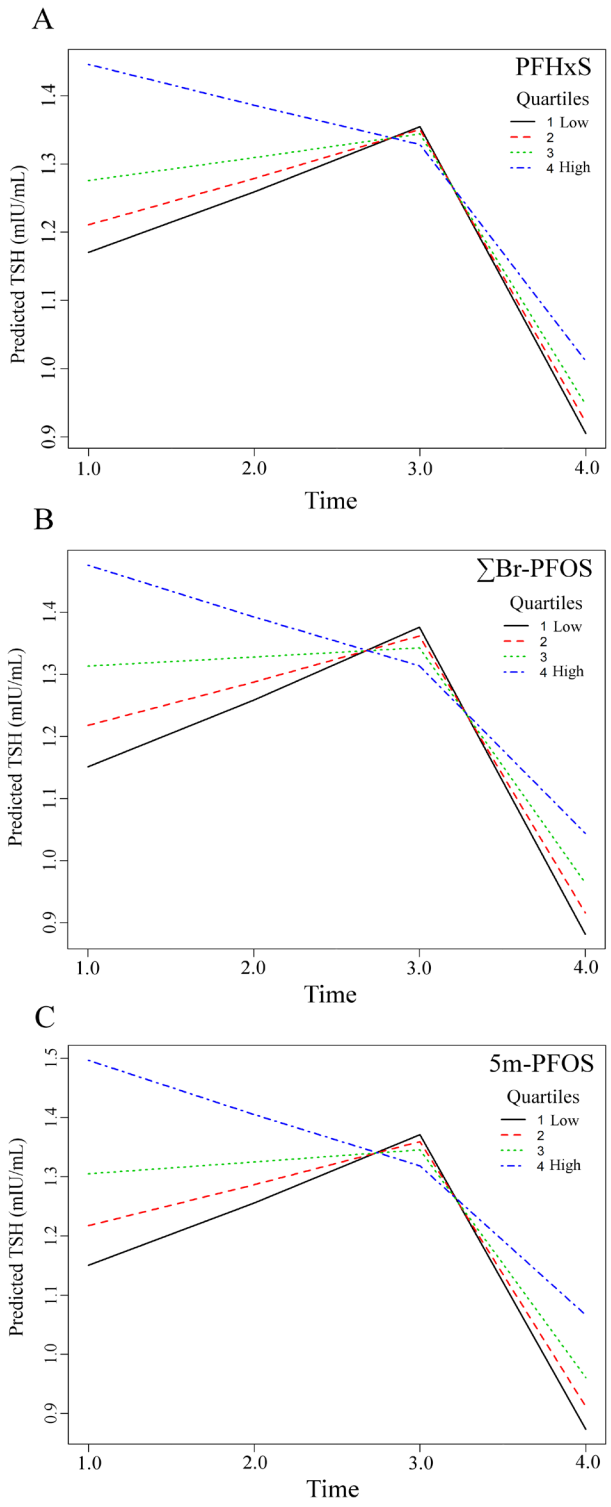

Figure 3-1. Change in predicted TSH (mIU/L) with each median quartile concentration (ng/mL) of PFHxS (A), ∑Br-PFOS (B), and 5*m*-PFOS (C), with binary predictors at a fixed reference level and at mean maternal age

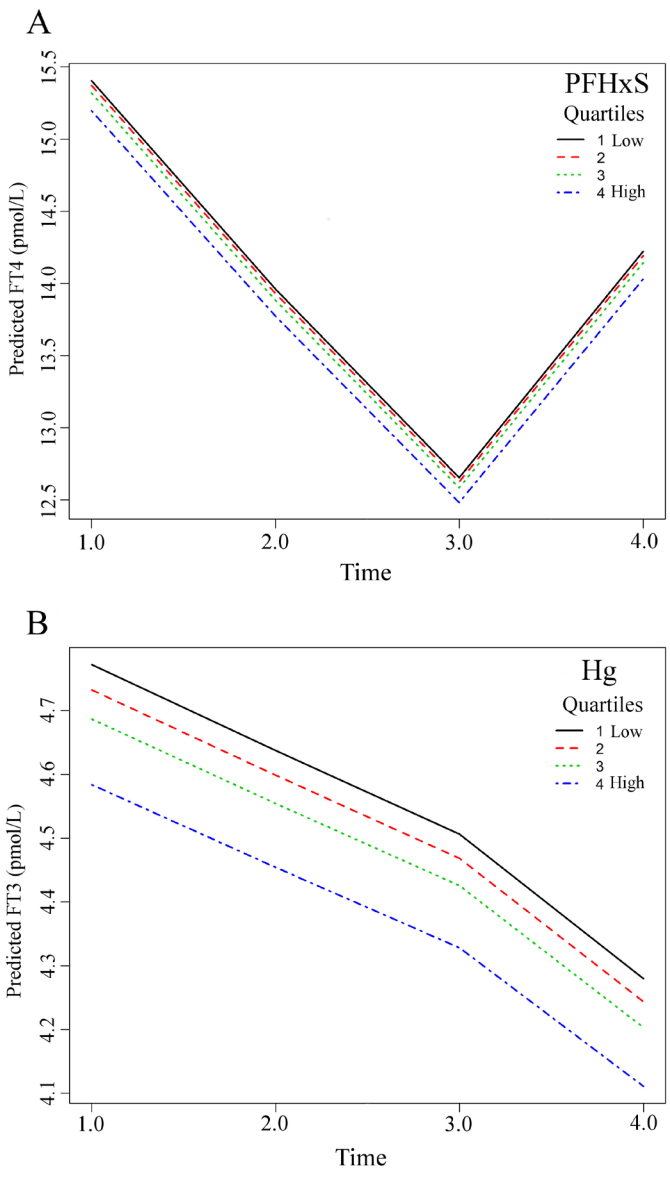

Figure 3-2. Change in predicted FT4 (pmol/L) with each median quartile concentration (ng/mL) of PFHxS (A), and the change in predicted FT3 (pmol/L) with each median quartile concentration (µg/L) of total Hg (B), with binary predictors at a fixed reference level and at mean maternal age

Chapter 4

Maternal Exposure to Perfluoroalkyl Acids (PFAAs) and Mercury (Hg) During Pregnancy is Associated with Neurodevelopment of Children from the APrON Birth Cohort

4.1 Introduction

Perfluoroalkyl acids (PFAAs) are a class of synthetic organic contaminants with a long history of commercial and industrial applications, including as stain and oil repellant coatings, in food packaging, and as a component of aqueous film-forming foams for fire-fighting[1]. Their inherent chemical stability and resistance to biodegradation has led to their ubiquitous presence in wildlife and the environment[2,3]. There are numerous sources of human exposure to PFAAs or their precursors, including their presence in carpeting, indoor air and household dust[4–8], but dietary intake is widely recognized as the major source of human PFAA exposure[9–11]. The most prominent PFAAs, perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS), perfluorooctanoate (PFOA) and perfluorononanoate (PFNA) are detectable in > 95 % of serum samples from Canada[12] and the United States[13].

Studies of matched maternal-child samples have reported detectable levels of PFAAs in umbilical cord blood[14–17], and in-vitro models[18] confirm the efficacy of PFAAs to cross the placenta. Not all PFAAs cross to the same extent, with branched isomers of PFOS crossing with greater efficiency than linear PFOS, resulting in fetal exposure to a different mixture of PFAAs than the mother[14,15,18]. These data raise questions about the possible effects of maternal PFAA exposure during pregnancy, as during critical periods of development, the fetus is known to be susceptible to endocrine disrupting chemicals in the gestational environment[19].

Evidence is growing that higher maternal serum concentrations of PFAAs are associated with altered thyroid hormone status in pregnancy (for review see[20]). A specific recurring observation in past studies has been higher maternal levels of thyroid stimulating hormone (TSH) among those with higher serum PFOS[21–24], consistent with findings reported in Chapter 3 for pregnant women in the APrON cohort. However, more detailed analytical chemistry of PFOS in serum using an isomer-specific method revealed that, despite making up a smaller proportion of total PFOS in maternal serum (i.e., ~30 % of total PFOS), total branched PFOS isomers (∑Br-PFOS) were positively associated with increased TSH, whereas corresponding results for linear

PFOS or total PFOS showed no significant association. High TSH levels with thyroxine (T4) within reference range is an indication of maternal hypothyroidism, a condition that has been associated with adverse birth outcomes[25–27] and decreased intelligence scores among children[28,29]. PFAAs have also been studied in association with maternal hypothyroxinemia, defined by low free T4 (FT4) concentrations (e.g., lowest 10th percentile) and levels of TSH within the normal range[30] that have been associated with decreased fetal neurodevelopment[31].

According to a recent review by Liew et al. few studies have investigated associations between prenatal PFAA exposure and developmental outcomes in early childhood (i.e., 6 months to 2 years)[32]. Moreover, among these studies, there is a lack of consistency between findings. For example, in Japan, PFOA was negatively associated with mental scales at 6 months but not at 18 months[33]. In Taiwan, PFOS was negatively associated with motor development of 2-year-old infants[34], but no significant associations were observed between either PFOS or PFOA and development milestones in Denmark[35]. To date, only the two most prominent PFAAs in human blood (PFOS or PFOA) have been considered, and related isomers have been ignored.

In older children (i.e. ages 4 to 18), cross-sectional studies have reported links between child exposure to multiple PFAAs with increased incidence of ADHD and impulsivity, including PFOS, PFOA, PFHxS, and PFNA, as well as perfluorodecanoate (PFDA) and PFOS precursor perfluorooctane sulfonamide[36–38]. However, longitudinal cohort studies of prenatal exposure did not reinforce these findings with respect to ADHD diagnosis[39,40] but also had smaller sample sizes, n = 206[40] and n = 215[39] that may have resulted in a lack of statistical power to detect weaker associations, especially for Liew et al. that was further limited by a small female subgroup (n = 41)[39]. These results provide indications that maternal PFAA exposure may affect child development, but further studies are needed to provide better understanding[32,41].

In Canada, pregnant women are advised to consume fish for the known nutritional benefits, but to limit intake of certain fish species with high mercury (Hg) content[42]. Populations with higher dietary intake of fish and seafood can have higher Hg blood levels[43], putting them at increased risk of developmental neurotoxicity during pregnancy[44]. Fish- and seafood-consuming populations also have higher exposure to PFAAs[45–47], thereby representing a potential co-exposure scenario for PFAAs and Hg. Recent animal developmental toxicology studies have included the co-exposure of methylmercury (MeHg) with various organic contaminants[48–53]. In

addition to our experimental co-exposure investigation of PFOS and MeHg (Chapter 2), one other study considered MeHg and PFAA co-exposure using PFOA[54]. In both cases, MeHg co-exposure with either PFOA or PFOS significantly delayed neurological development of newborn rat pups and demonstrated chemical antagonism in behavioural testing of juvenile animals. We hypothesized that co-exposure of PFAAs and Hg could lead to confounding or toxic interactions, and in the current study we test this by including maternal Hg exposure in models of PFAAs and child neurological development.

The aim of the current investigation was to examine associations between maternal exposure to PFAAs and developmental outcomes measured in 2-year-olds using the Bayley scales of infant and toddler development, 3rd edition (Bayley-III) among participants of a Canadian pregnancy cohort study known as the Alberta Pregnancy Outcomes and Nutrition (APrON) cohort. Exposure to total Hg (THg) and a wide range of PFAAs was considered, from the 4-carbon molecule perfluorobutanoate (PFBA) to the 14-carbon molecule perfluorotetradecanoate (PFTeA). An isomer-specific analysis method was used to distinguish individual branched isomers of PFOS and PFOA.

4.2 Methods[XI]

4.2.1 Study Participants and Sample Collection[XII]

APrON participants were recruited and provided informed consent between March 2009 and July 2012. A full description of rationale, methods, and data on diet, physical activity, mental health, medical history, and demographics has been published elsewhere[55,56]. All study protocols were approved by the University of Calgary Health Research Ethics Board and the University of Alberta's Human Ethics Research Board. The current study included a subset of women from APrON (n = 494) residing in Calgary that provided a blood sample during the second trimester (14 - 26 weeks gestation). Inclusion criteria included recruitment prior to 18

[XI] I was responsible for experimental design and analysis of PFAAs in maternal plasma from APrON participants using protocols detailed in Chapter 3.

[XII] Participant recruitment, and data and sample collection were conducted by members of the APrON team at the Alberta Children's Hospital (University of Calgary)

weeks gestation (first visit mean gestation = 14.3 weeks) and conceived naturally without the use of fertility hormones or assisted reproductive techniques. Data were collected through in-person interviews administered as a first-visit questionnaire and a follow-up questionnaire at the time of biological sample collection.

For this investigation, potential covariates and confounders included maternal age, education, household income, ethnicity, parity, medical conditions, body mass index (BMI), as well as smoking, alcohol, and recreational drug use. Participants were excluded if children were identified to have a genetic disorder that would impact development outcomes (n= 4). Detailed information for selected covariates among maternal child pairs in the final study subset (n = 490) are presented in Table 4-1.

4.2.2 Analysis of PFAAs

A total of 25 PFAA analytes, including 16 perfluoroalkyl carboxylates (11 linear and 5 branched isomers) and 9 perfluoroalkyl sulfonates (4 linear and 5 branched isomers) were monitored in maternal plasma samples collected during the second trimester of pregnancy. Information on sample preparation, extraction, and analysis of PFAAs is detailed in Chapter 3. Briefly, PFAAs in plasma spiked with mass-labeled internal standard were extracted using protein precipitation with acetonitrile[57], followed by dispersive cleanup using bulk graphitized carbon[58]. PFAAs were primarily extracted and analyzed in the plasma fraction of whole blood, but serum was utilized when plasma was not available (14 % of all samples).

Isomer-specific PFAA analysis was by high performance liquid chromatography-triple-quadrupole mass spectrometry (HPLC-MS/MS). An external solvent-based calibration curve was utilized, and each linear and branched PFAA was quantified using the appropriate mass labeled internal standard (Chapter 3). All collection and storage materials were tested for background contamination using HPLC-grade water as a surrogate matrix, and no contamination was detected in any materials used in lab procedures or for sample collection and storage.

4.2.3 Analysis of Mercury[XIII]

Total Hg (THg) was analyzed from the red blood cell (RBC) fraction of whole blood from samples collected during the second trimester of pregnancy. Information on sample preparation and analysis of maternal Hg is also detailed in Chapter 3. Briefly, the RBC fraction was removed from whole blood samples after centrifugation. Prepared RBC samples were analyzed for total Hg by ICP-MS/MS. Complete details of QA/QC, including instrumental limits of detection and quantitation (LOD, LOQ) and THg recovery are described in Appendix B.

4.2.4 Assessment of Neurodevelopment[XIV]

Child neurodevelopment was evaluated at approximately 2-years of age using the Bayley-III. Testing was conducted by a team of psychometrists under the supervision of a registered psychologist at the Alberta Children's Hospital (Calgary, AB). The Bayley-III is a comprehensive assessment of early childhood development that includes five major scales (and subsets), including *Cognitive* (cognitive scale of 91 items), *Language* (expressive and receptive communication) and *Motor* (fine, and gross motor skills) scales assessed by the examiner; whereas primary caregivers complete a *Social-Emotional* Questionnaire (social development and related behaviours), and estimates of *Adaptive Behaviour* (functional areas of communication, community use, health and safety leisure, self-care, self-direction, pre-academics, home living, and social and motor subsets)[59]. The composite scores of the five major scales were used to interpret child neurological development.

4.2.5 Statistical Analysis[XV]

Multiple linear and curvilinear regression models were used to determine associations of prenatal exposure to PFAAs and Hg on child neurological development. All statistical analyses were performed using R (v3.3.2) with alpha set to 0.05 for determination of statistical

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[XIII] Mercury analysis was conducted by Dr. Amy MacDonald, and Dr. David Kinniburgh of the Alberta Centre for Toxicology (University of Calgary)

[XIV] Neurodevelopment scoring of 2-year old children provided by APrON team members led by Dr. Deborah Dewey at the Alberta Children's Hospital (University of Calgary)

[XV] Statistical analysis was conducted by Morteza Hajhosseini and Dr. Irina Dinu of the School of Public Health (University of Alberta)

significance. Concentrations of predictors were log-transformed to account for skewed distribution of PFAA exposure, and concentrations below LOD were treated according to Hornung and Reed, 1990 and assigned a value of (LOD/√2)[60]. A quadratic equation was used to explore non-linear associations of PFAAs or THg with neurodevelopment outcomes. The data were mean-centered prior to interpretation of terms in the quadratic equation (Eq. 1).

Eq. 1:

$$y = a + bx + cx^2$$

Interpretation of the quadratic term was dependent on the direction of the linear term (b) and the ratio of the linear to quadratic term, as half of this ratio represents the point on the x-axis (PFAA concentration) where the slope levels off. For example, a positive linear coefficient and negative quadratic coefficient representing the association of PFAA with neurodevelopment outcome; the calculated value represents the point that the slope would level off at a maximum and then decreases based on the value of the quadratic coefficient (Eq. 2). For purposes here, this calculated value represented the threshold concentration (of PFAA or THg) at which the quadratic association became significant ($p < 0.05$).

Eq. 2:

$$y = \left(-\frac{b}{2c}\right) + \bar{x}$$

For potential confounders, the following variables were considered: maternal ethnicity, education level, household income, parity, body mass index, smoking, use of alcohol or recreational drugs during pregnancy. Infant sex, gestational age at birth (weeks), age at testing (months), as well as the gestational age at time of maternal sample collection were also considered. Each potential covariate (Table 4-1) was evaluated in a univariate model in the presence of the main predictor, and covariates with a level of significance of $p < 0.2$ were adjusted for in the multiple model. Multicollinearity between covariates was tested and the

goodness of fit for all the models was evaluated exploring the models' residuals. Evaluation in statistical models was restricted to PFAAs that were detected in > 50 % of samples.

Within the model, the main effect linear and quadratic estimates (β) were determined, described as the change in outcome per unit change in the predictor (PFAA or THg) when significant covariates from the first step were adjusted for. The significance of the interaction of child sex ($p < 0.05$) was also considered, if the interaction was significant, a separate model was used to assess the stratum-specific effect of infant sex (i.e., boys and girls). To address the additional hypothesis of contaminant co-exposure, the significance of the interaction of THg co-exposure ($p < 0.05$) was also considered to determine its influence on associations of maternal PFAA exposure and infant neurodevelopment.

4.3 Results

4.3.1 Population Description

Maternal data were collected from first visit questionnaires, and infant data from hospital birth records of participants residing within the metropolitan area of Calgary, Canada (Table 4-1). Mothers had a median age of 32, were primarily Caucasian (84 %), highly educated (71 % completed post-secondary education), with a family income >$77K CAD (78 %). Most were nulliparous or primiparous (87 %), non-smokers or quit when trying to become pregnant (94 %), and during pregnancy did not consume alcohol (91 %) or use recreational drugs (96 %). The median gestational age at birth was 39.0 weeks, and median child age at testing was 30.0 months. The proportion of child sex was 49 % male and 51 % female.

4.3.2 Maternal PFAA and THg Exposure

PFAAs in maternal plasma (PFOA, PFNA, PFDA, PFHxS, and PFOS) were detectable in > 99 % of samples (Table 4-2). Major PFAAs, such as PFOA, PFOS and PFHxS had the highest geometric mean (GM) concentrations in maternal plasma at 2.13, 3.45, and 1.02 ng/mL respectively, followed by 0.74 ng/mL for PFNA. The sensitivity of the current method allowed for detection (% detection frequency) and quantitation (GM) of other PFAAs that are typically excluded in cohort studies, including: 0.26 ng/mL PFDA (100 %), 0.16 ng/mL PFUnA (89 %),0.04 ng/mL PFHpA (67 %), and 0.04 ng/mL PFDoA (56 %) (Table 4-2). Spearman

correlations indicated that individual perfluoroalkyl carboxylates were moderately to highly intercorrelated (rho = 0.22 – 0.81, $p < 0.05$), and weakly correlated with total PFOS (rho = 0.1 – 0.3, $p < 0.05$). PFHxS was also moderately correlated with total PFOS (rho = 0.46, $p < 0.05$), but not with any carboxylates (Appendix C).

Concentrations for the most PFAAs in serum of mothers giving birth to either boys or girls were similar (Table 4-2). However, significantly higher concentrations of total PFOS were detected in women who gave birth to boys (GM = 3.66) compared to women giving birth to girls (GM = 3.26) based on a Student t-test ($p < 0.05$). These findings are in agreement with Mondal et al. who reported higher child to mother ratios of PFOA (GM = 1.25 and 1.05) and PFOS (GM = 1.51 and 1.35) in serum of boys and girls, respectively[61].

Linear and branched isomers of PFOS were detected in > 97 % of maternal plasma samples (Table 4-3). GM concentrations (ng/mL) were highest for the linear-PFOS (2.31, 69 % of total PFOS) over the sum of branched PFOS isomers (1.08, 31 % of total PFOS), including: 1*m*-PFOS (0.07), ∑3*m*+4*m*-PFOS (0.24), 5*m*-PFOS (0.33), and *iso*-PFOS (0.43). All PFOS branched isomers were moderately to strongly intercorrelated (rho = 0.580 – 0.930, $p < 0.05$) (Appendix C). Similar to total PFOS, all PFOS isomers except for 1*m*-PFOS were significantly higher in women who gave birth to boys (Table 4-3). For PFOA, only the linear isomer (2.08 ng/mL) and *iso*-PFOA (0.08 ng/mL) were detected, and *iso*-PFOA was excluded from analysis in multiple regression models due to its low detection frequency in the sample population (< 50 %). Therefore, all further mention of PFOA refers exclusively to linear-PFOA.

Maternal THg concentration (GM) in the RBC fraction was 0.94 µg/L, detected in > 97 % of samples (Table 4-2). THg levels in the RBC fraction are approximately two-fold higher than if whole blood had been analyzed, and by accounting for this difference the THg concentrations here are comparable to whole blood measurements in pregnant women among 10 cities in Canada[62]. Nevertheless, THg levels here are lower than in Cree First Nations in Canada where higher exposure was significantly correlated with increased fish consumption[63].

4.3.3 Child Neurodevelopment Assessment

Girls performed significantly higher than boys in composite scores of cognition, language, social-emotional development, and general adaptive behavior ($p < 0.05$) on the

Bayley-III (Table 4-4). Higher performance in girls has been reported before across all scales of the Bayley-III[64], as well as in mental index scales for earlier versions of the Bayley assessment[65]. An interaction term for infant sex was included in multiple models to address these differences, and when a significant interaction was observed, a separate sex-stratified analysis was conducted.

4.3.4 Association of Maternal PFAA Exposure with Child Neurodevelopment

Increasing composite scores from each of the Bayley-III scales of cognition, language, motor, social emotional and general adaptive behaviour are classified as higher measures of child performance (i.e., extremely low, borderline, low average, average, high average, superior, and very superior). In adjusted models, higher maternal PFHpA and PFDoA concentrations were negatively associated ($p < 0.05$) with cognition composite scores (Table 4-5). The cognition scale includes measures of information processing, speed of processing, and problem solving in early development[66]. PFHpA was also negatively associated with composite scores of social-emotional outcomes ($p < 0.05$), which evaluate the ability of the child to use emotions and expressions through words or symbols, as well as comprehension of emotional cues and signals[67]. PFOS and PFHxS were positively associated with composite scores of general adaptive behaviour. No PFAAs, nor Hg were significantly associated with motor development.

Exploratory analysis of the relationships of PFAAs and Hg with composite scores from the Bayley-III revealed non-linear relationships that were similar to a previous investigation associated child Bayley outcomes with maternal manganese exposure[68]. Here, a quadratic term to account for any significant non-linear association was included in multiple models of maternal PFAA, and THg exposure with child outcomes from the Bayley-III (Table 4-5).

PFOS was significantly associated with child outcomes from the language scale (Table 4-5), but unlike all other outcomes from the Bayley-III, only the quadratic coefficient was significant ($p < 0.05$) for language scores. For example, increasing exposure to PFOS was not associated with language scores up to a calculated threshold of 4.72 ng/mL (Eq. 2), but then significantly negatively associated with language scores at higher PFOS concentrations ($p < 0.05$). Of the PFOS isomers, linear-PFOS, 1*m*-PFOS, had significant quadratic coefficients ($p < 0.05$) associated with language scores (Table 4-6). linear-PFOS and 1*m*-PFOS isomers were

negatively ($p < 0.05$) associated with language scales above thresholds of 3.78 and 1.70 ng/mL respectively. Unlike linear-PFOS and 1*m*-PFOS, the *iso*-PFOS isomer had a significant linear coefficient ($p < 0.05$), resulting in a positive association with language scores across the entire range of exposure.

Stratifying the population by sex indicated that PFHxS, total PFOS and total branched PFOS isomers were positively associated with general adaptative behaviour scores in females ($p < 0.05$), but not males (Table 4-7). These results reveal a sex-specific association for PFAAs, only associated with general adaptive behaviour in girls, specifically with increased scores in areas of functional pre-academics, home-living and community use. These results should be interpreted with caution because general adaptive behaviour screening is conducted by the primary care-giver and may be subject to scoring bias.

4.3.5 Association of Prenatal THg Exposure with Child Neurodevelopment

THg was positively associated ($p < 0.05$) with Bayley-III composite scores of child language (Table 4-5). Like with PFAAs, only the quadratic coefficient was significant ($p < 0.05$). THg was positively associated with child language above a threshold of 5.38 µg/L (Eq. 2). However, it should be noted that this directional change takes place at Hg levels that are outside the range of the current dataset (Table 4-2), and the effect size indicates a relatively low change in score per unit change in Hg (quadratic $\beta = 0.4$), suggesting that this finding may be a spurious result and not of clinical significance.

4.3.6 Interaction of THg Co-Exposure on Associations of PFAAs and Child Development

It was hypothesized Hg co-exposure alters the effects of PFAAs on child neurodevelopment, either as a confounder in models or by toxicological interaction. Regarding confounding, except for a moderate correlation between THg and PFUnA (rho = 0.4, $p < 0.01$), Hg was not correlated with any PFAA analyte, and thus was not considered a confounder in the current study (Appendix C). Nevertheless, confounding by THg and PFAA co-exposure may be more relevant in populations with higher dietary intake of fish. Inclusion of THg interaction (Table 4-8) did not change significant associations of PFHpA with language or social emotional scores, or *iso*-PFOS with language scores previously mentioned (Table 4-5), suggesting that these PFAAs are significantly associated with child neurodevelopment and unaffected by the

presence of THg. However, previously significant associations of PFDoA with cognition, PFHxS with general adaptive behaviour, total PFOS with language and general adaptive behaviour, and isomers linear-PFOS and *iso*-PFOS with language scores (Table 4-5) were nullified when THg was included as an interaction term (Table 4-8). These results indicate that co-exposure to different classes of contaminants have the potential to induce significant changes in associations of child neurodevelopment outcomes, as effects may be potentiated, masked or nullified with the inclusion of additional interacting contaminants.

Maternal PFNA concentration, not associated with child neurodevelopment (Table 4-5) became significant ($p < 0.05$) when including THg that was negatively associated with both child motor and social emotional behaviour scores (Table 4-8). This result suggests that neither PFNA or THg concentrations during pregnancy modeled individually were sufficient to have an effect on child development, but modeling PFNA with the combined interaction of THg revealed significant negative associations. Although including THg in PFAA-neurodevelopment models changed the main effects of PFAAs on child neurodevelopment outcomes, the PFAA-THg interaction term was not statistically significant in any of these models.

4.4 Discussion

In this birth cohort study, PFAA exposure was negatively associated with neurodevelopment of 2-year-old children after adjusting for significant covariates. Higher prenatal exposure to PFHpA and PFDoA were significantly associated with decreased child performance on the cognition scale of the Bayley-III (Table 4-5). Previous studies that primarily focused on the dominant PFAAs, PFOS and PFOA, have also reported that PFAAs are associated with adverse neurological development (see Liew et al. for review[32]). For example, prenatal PFOA exposure was associated with decreased mental developmental indices in 6-month-old girls using the Bayley Scales of Infant Development 2nd edition[33], and higher PFOS in cord-blood was negatively associated with whole test outcomes, particularly for gross-motor subdomains[69]. However, other investigations have not found strong associations between PFOS or PFOA and neurobehavioural outcomes[70,71] or development milestones[35]. Numerous factors, including sample size, infant age at testing, different sample matrices (e.g., maternal serum, cord blood, or breast milk), and the level of exposure may account for some variability in the level of significance between studies. For example, for both significant studies with PFOA and PFOS,

sample sizes were relatively small (n= 178, and 239, respectively)[33,69] and for one instance, these associations were no longer significant when children were retested at 18-months-old[33].

Maternal PFOS (GM = 3.45 ng/mL) and PFOA (GM = 2.13 ng/mL) in second trimester samples were comparable to values from recent cohort studies in Canada (MIREC)[72], Japan (Hokkaido)[33], and Spain (INMA)[73]. Decreasing temporal trends of PFAAs have been observed in the general population since phase-outs of PFOA, PFOS and its precursors by 3M Co. (decreased PFOS (75%) and PFOA (46 %) from 2001 to 2010)[13,74]. PFAA values from biomonitoring in recent cohorts such as our own have lower maternal plasma levels when compared to cohort samples collected in the early 2000s (e.g., 24.9 ng/mL PFOS and 5.60 ng/mL PFOA from project VIVA, USA[75], and 28.1 ng/mL PFOS and 4.28 ng/mL PFOA from the DNBC, Denmark[76]. Decreased exposure may be an important factor contributing to the significance of findings for PFOS and PFOA in various cohort studies.

Higher prenatal concentrations of PFHpA were significantly associated with decreased scores on the social-emotional scale of the Bayley-III (Table 4-5). Unlike PFOS and PFOA, minor and lesser studied PFAAs such as PFHpA and PFDoA have been not routinely considered in epidemiological studies of prenatal PFAA exposure and early child neurodevelopment[33,35,69,70], often excluded from statistical analyses due to lower detection frequencies in maternal plasma and umbilical cord blood using traditional HPLC-MS/MS methods[77]. However, modified approaches such as LC-column switching[78], or an alternative HPLC column stationary phase, such as the one used here (i.e., pentafluorophenylpropyl, (PFP)) over traditional C8 or C18 phases provide increased sensitivity and better separation of PFAAs from co-eluting interferences[79]. Although PFOA and PFOS represent the most prominently detected PFAAs, future studies would benefit from expanding their scope of analysis to include lesser studied PFAAs and corresponding branched isomers.

Non-linear relationships were observed from exploratory data analysis of the relationships of PFAAs and branched PFOS with language scales (Tables 4-5 and 4-6). Although a few studies, primarily of prenatal manganese exposure have considered non-linear associations with measured indices of child neurodevelopment[68,80,81], only a few studies have utilized this approach for observing the associated effects of PFAAs[37,75]. In school-age children, the association of PFOA and incidence of attention deficit/hyperactivity disorder (ADHD) had an

inverted J-shape but was considered a spurious finding, as differences in regional geography (e.g., schools in non-PFOA contaminated districts also had increased ADHD diagnoses) and insufficient data were available to confirm this association[37]. A more recent study used cubic regression splines to fit non-linear relationships of increased prenatal PFOA, PFOS and PFHxS exposure with decreased visual-motor scores mid-childhood[75].

In toxicology, threshold values are routinely considered to identify the lowest observed adverse effect level (LOEAL): the concentration at which a chemical or contaminant elicits an adverse effect, and exposure below this threshold is unlikely to induce risk to health and development. Incorporating a quadratic term into regression models allows for the opportunity to identify thresholds in the context of epidemiology, and thus, estimate the lower concentration range of predictors (PFAAs and THg) where associations might be null. For example, the association for total-PFOS was only significant ($p < 0.05$) with language scale above 4.72 ng/mL, an association that may have been overlooked using general linear models. Applying a linear approximation in regression models is common practice and generally accepted in epidemiological studies. However, limiting the analysis to linear models when data patterns exhibit non-linear relationships (e.g., U- or J-shaped associations of contaminant exposure with health related outcomes) may lead to false negatives (i.e., non-significant associations)[82].

This is the first epidemiology study of PFAAs to consider isomer-specific associations with child neurodevelopment. Branched PFOS isomers were detectable in 97-100 % of samples, and increased concentrations of linear- and 1*m*- PFOS were negatively associated with language scales of child development, but only above a threshold (Table 4-6). In Experimental animal models, branched isomers of PFAAs exhibited different toxicokinetic properties[83]. Branched PFOS was present in increased proportions in umbilical cord blood compared to paired maternal samples, suggesting increased transplacental transfer of branched isomers[14,15]. In Chapter 3 it was shown that PFOS isomers were associated with altered maternal thyroid hormone homeostasis, providing a potential mechanism for PFAA-mediated adverse child neurodevelopment. Furthermore, various global populations have been shown to have different proportions of branched PFOS isomers. For example, populations in China (52 %)[84] and Norway (30-50 %)[11,85] had higher proportions of branched PFOS isomers compared to current results in Canada (31 %) (Table 4-3). These results suggest that during pregnancy, prenatal exposure to branched PFOS isomers may be a risk factor for alteration of normal neurodevelopment.

Infant sex was a significant effect modifier for the association between PFOS and scales of general adaptive behaviour (Tables 4-5 and 4-6). A further stratified analysis found sex-specific differences, as higher PFOS in girls was positively associated with measures of adaptive behaviour that was not present in boys (Table 4-7). This suggests an apparent PFOS-associated increase in performance of girls in their ability to acquire a skillset including aspects of "communication, social, motor, health and safety, home living, self-care, leisure, community use, functional pre-academics, leisure, and self-direction"[86]. However, the questionnaire for general adaptive behaviour is administered by the primary caregiver, and these results should be interpreted with caution as outcomes may be subject to reporting bias that overestimate scoring responses[87].

Models of human exposure to individual contaminants are routinely employed in large-scale epidemiology studies, but co-exposure and the presence of chemical mixtures are the realities in many human circumstances. Including THg as an interaction in regression models revealed significant ($p < 0.05$) negative associations between PFNA and subscales of motor and social-emotional measures (Table 4-8) that were not detected in main effect models of either PFNA or THg alone. Past studies have investigated mixtures of heavy metals (arsenic, manganese, and lead) on child neurodevelopment in highly exposed populations[81,88]. Exposure to arsenic was found to act as a "potentiator" of manganese (i.e., the associated toxicity of manganese was dependent on the presence of arsenic) but neither contaminant alone was sufficient to induce a significant main effect[88]. THg co-exposure may similarly increase the sensitivity of language and social-emotional development to PFNA.

The significant chemical-chemical interactions detected here, combined with findings from previous investigations of heavy metal mixtures reinforce the value of more refined approaches to identify the effects of prenatal exposure to chemical mixtures. Multiple regression models utilizing chemical concentrations are limited to interpretation of individual and co-exposure scenarios, while increasing the number of chemicals into statistical models becomes increasingly complex. For example, the INMA cohort included up to 81 different environmental exposures[89], and there was difficulty linking specific associated outcomes with specific contaminants. Recent approaches to multi-pollutant assessment of contaminant mixtures have used principle component analysis (PCA) to reveal the "exposome" during pregnancy, thereby identifying groups of organic pollutants and heavy metals that exhibit similar patterns of

exposure[89–91]. One investigation used PCA plots to evaluate multiple chemical exposures, establishing scores of chemical mixtures for inclusion in regression models and examining associations with increased body mass index in 7-year-old children[92]. PCA plots are a powerful tool in identifying patterns of contaminant exposure that may be subsequently combined with linear regression and ANOVA to determine if groups or specific mixtures of contaminants are associated with various health outcomes. In my own findings, PFAAs were not highly correlated with THg (Appendix C), and few associations of PFAAs with child neurodevelopment scores were altered by the interaction of THg (Table 4-8). Nonetheless, results with THg should still be considered, especially in populations with increased THg exposure (i.e higher fish consumption). Furthermore, underlying influence from additional contaminants may also be occurring but were not accounted for as they were not measured in the current population.

The strengths of this study lie in the relatively large sample size (n = 490) and detailed questionnaires in the APrON cohort that allowed for adjustment of numerous potential confounders (listed, Table 4-1). Cognitive, language and motor scales were conducted by trained psychometrists blind to the exposure status of participants. Furthermore, when appropriate, quadratic terms were ultilized to more accurately model associations of maternal PFAA exposure with specific aspects of child development (e.g., language scores), The effects of chemical mixtures were also examined by considering the interaction of THg. PFAAs were primarily measured in samples collected in the second trimester, which is appropriate given the long half-life of PFAAs in human (e.g., PFOS = 5.4 years, and PFOA = 2.3 years)[93] and previous findings of highly correlated exposure across trimesters of pregnancy.[72] Although relatively short when compared to PFAAs, THg was also measured in the second trimester and (although shorter than PFAAs) has a relatively long half-life in humans (~ 44 days). Thus, the current study had robust measures of prenatal PFAA and THg exposure.

The Bayley-III scales of infant and toddler development is a widely accepted method of assessing the progression of child neurological development[59]. However, a review of the Bayley-III revealed cognition, language, and motor development subscales had a tendency to overestimate child performance, resulting in an underestimation of developmental delay in 2 year old children[94]. Although this finding has clinical implications in identifying children with developmental delays, it should not influence the power to determine associations between contaminant exposure and these endpoints in a background population.

Although this is one of the largest detailed investigations of PFAAs on early child neurodevelopment, this study was nevertheless subject to some limitations. Child assessment of neurodevelopment took place at 2-years, however, longitudinal studies have shown that neurodevelopment outcomes at one age do not necessarily hold at other ages[95], and most studies of PFAA exposure have focused on outcomes in older children (e.g., 4 to 18 years old)[32,41]. To address this, the APrON cohort conducted follow-up assessments of the same children at 3 and 5 years of age, and the trajectory of development is planned for future analyses in this cohort. The participants in the current study represent a very specific demographic (primarily Caucasian, highly educated, and with relatively high household income). Although such bias, which results in less variation in participants, may be considered an asset for interpretation of the results, it is not necessarily a true representation of the Canadian population in general. Finally, samples collected at birth (e.g., umbilical cord blood) provide a better indication exposure to PFAA isomers.

4.5 References

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Table 4-1. List of covariates evaluated in univariate models from mother-child participants of the APrON cohort, considered for inclusion in multivariate models

	Category	(n)	Median	Range
Maternal age (Continuous)	-	490	32.0	21.0 – 39.0
Maternal Sample collection – gestation week (Continuous)	-	490	17.3	14.1 - 32.0
Maternal education (Categorical)	< high school	4	-	-
	High school	28		
	Technical school	83		
	University	231		
	Post-grad	122		
	Missing data	22		
Maternal ethnicity (Categorical)	Caucasian	417	-	-
	Chinese	16		
	Filipino	3		
	Japanese	2		
	Korean	0		
	Latin American	13		
	Native/Aboriginal	1		
	South Asian	7		
	South East Asian	4		
	Arab	2		
	West Asian	0		
	Black	2		
	Other	5		
	Missing data	18		
Household Income (Categorical)	< 20K	8	-	-
	20K to 39,999	21		
	40K to 69,999	52		
	70K to 99,999	105		
	100K and above	282		
	Missing data	22		
Parity (Categorical)	no children	266	-	-
	1 previous child	162		
	2 previous children	41		
	3 previous children	5		
	Missing data	16		
Smoking during pregnancy (Categorical)	Yes	13	-	-
	No	462		
	Missing	15		
Alcohol use during pregnancy (Categorical)	Yes	27	-	-
	No	449		
	Missing	14		
Recreational drug use – history (Categorical)	Yes	4	-	-
	No	472		
	Missing	14		

Maternal pre-pregnancy body mass index (continuous)	-	490	23.6	18.6 – 38.6
Child age at birth – weeks (Continuous)	-	-	39.0	34.9 – 41.4
Child age at testing – months (Continuous)	-	-	30.0	26.0 – 32.0
Infant sex (Categorical)	Male Female Missing	238 252 1	-	-

Table 4-2. Descriptive statistics of maternal exposure to PFAAs (ng/mL) and mercury (µg/L) of participants in the Bayley-III

	DL (% >)	AM	GM (SD)	Percentile				
				5th	25th	50th	75th	95th
All Children								
PFHpA	67	0.149	0.041 (0.332)	< DL[A]	< DL[A]	0.048	0.107	0.788
PFOA	100	2.90	2.13 (3.40)	0.711	1.33	2.13	3.19	7.48
PFNA	99	1.05	0.742 (1.28)	0.295	0.481	0.695	1.13	3.06
PFDA	100	0.384	0.263 (0.514)	0.073	0.170	0.253	0.391	1.15
PFUnA	89	0.202	0.163 (0.147)	0.071	0.085	0.167	0.265	0.464
PFDoA	56	0.051	0.035 (0.056)	< DL[A]	< DL[A]	0.018	0.071	0.142
PFHxS	100	1.54	1.02 (1.77)	0.243	0.577	1.036	1.745	4.85
Total PFOS	100	3.96	3.45 (2.08)	1.49	2.29	3.63	5.04	8.13
Hg	97	1.47	0.984 (1.31)	0.142	0.593	1.07	1.94	3.80
Boys (n = 252)								
PFHpA	68	0.154	0.041 (0.368)	< DL[A]	< DL[A]	0.049	0.108	0.781
PFOA	100	2.93	2.18 (3.17)	0.705	1.36	2.21	3.25	7.40
PFNA	98	1.04	0.737 (1.22)	0.291	0.481	0.712	1.13	2.96
PFDA	100	0.385	0.270 (0.458)	0.076	0.171	0.261	0.402	1.12
PFUnA	90	0.206	0.166 (0.148)	0.071	0.084	0.176	0.267	0.516
PFDoA	54	0.052	0.036 (0.053)	< DL[A]	< DL[A]	0.018	0.071	0.149
PFHxS	100	1.61	1.07 (1.75)	0.238	0.612	1.12	1.74	5.09
Total PFOS*	100	4.21	3.66 (2.17)	1.40	2.47	3.99	5.43	8.19
Hg	97	1.51	1.02 (1.41)	0.162	0.632	1.04	1.94	4.18
Girls (n = 237)								
PFHpA	64	0.146	0.041 (0.291)	< DL[A]	< DL[A]	0.048	0.104	0.787
PFOA	100	2.88	2.08 (3.65)	0.712	1.27	2.01	3.08	7.89
PFNA	100	1.06	0.751 (1.35)	0.301	0.484	0.665	1.11	3.41
PFDA	100	0.386	0.260 (0.570)	0.073	0.171	0.245	0.368	1.31
PFUnA	83	0.197	0.160 (0.148)	0.071	0.085	0.155	0.260	0.450
PFDoA	57	0.050	0.034 (0.060)	< DL[A]	< DL[A]	0.018	0.071	0.130
PFHxS	100	1.47	0.972 (1.81)	0.251	0.548	0.974	1.79	3.68
Total PFOS	100	3.69	3.26 (1.95)	1.57	2.20	3.34	4.52	7.53
Hg	99	1.42	0.94 (1.21)	0.122	0.526	1.15	1.92	3.57

Abbreviations: AM (arithmetic mean), GM (geometric mean), SD (standard deviation), DL (detection limit)
Values representative of Timepoint 2 (96 %) samples, values from Timepoint 1 (0.6 %), Timepoint 3 (3 %), and Timepoint 4 (0.4 %) were used when Timepoint 2 plasma was not available
[A] Value below method detection limits (MDL; ng/mL) detailed in supplementary material
* $p < 0.05$ level of significance, difference in maternal PFAA concentration (ng/mL) of women giving birth to boys or girls

Table 4-3. Descriptive statistics of maternal exposure to PFOS isomers (ng/mL) of participants in the Bayley-III

	DL (% >)	Total (%)	AM	GM (SD)	Percentile				
					5th	25th	50th	75th	95th
All Children									
Linear	100	68.8	2.72	2.31 (1.49)	0.957	1.56	2.50	3.41	5.64
∑-Branched	100	31.2	1.23	1.08 (0.661)	0.479	0.720	1.09	1.60	2.49
1*m*-	97	2.0	0.080	0.065 (0.058)	0.023	0.045	0.064	0.098	0.187
∑-3*m*+4*m*-	100	6.8	0.267	0.235 (0.144)	0.105	0.162	0.237	0.339	0.536
5*m*-	100	9.8	0.388	0.328 (0.234)	0.123	0.207	0.340	0.507	0.831
iso-	100	12.6	0.498	0.426 (0.284)	0.182	0.288	0.430	0.646	1.04
Boys (n=252)									
Linear	100	69.0	2.91	2.49 (1.57)	0.922	1.69	2.70	3.72	5.98
∑-Branched	100	31.0	1.31	1.14 (0.69)	0.475	0.803	1.24	1.69	2.53
1*m*-	96	2.0	0.083	0.066 (0.061)	0.022	0.051	0.068	0.101	0.184
∑-3*m*+4*m*-	100	6.8	0.285	0.249 (0.155)	0.104	0.173	0.255	0.355	0.569
5*m*-	100	9.9	0.416	0.351 (0.248)	0.124	0.221	0.369	0.537	0.847
iso-	100	12.4	0.524	0.443 (0.293)	0.180	0.309	0.470	0.678	1.08
Girls (n=237)									
Linear*	100	68.7	2.53	2.21 (1.39)	1.01	1.52	2.24	3.12	5.20
∑-Branched*	100	31.3	1.15	1.02 (0.623)	0.480	0.674	1.01	1.43	2.45
1*m*-	97	2.1	0.077	0.063 (0.054)	0.023	0.043	0.061	0.090	0.190
∑-3*m*+4*m*-*	100	6.7	0.248	0.222 (0.128)	0.111	0.155	0.218	0.311	0.492
5*m*-*	100	9.7	0.357	0.305 (0.216)	0.123	0.197	0.306	0.463	0.801
iso-*	100	12.8	0.470	0.410 (0.272)	0.187	0.277	0.386	0.591	1.03

Abbreviations: AM (arithmetic mean), GM (geometric mean), SD (standard deviation), DL (detection limit)
Values representative of Timepoint 2 (96 %) samples, values from Timepoint 1 (0.6 %), Timepoint 3 (3 %), and Timepoint 4 (0.4 %) were used when Timepoint 2 plasma was not available
[a] Value below method detection limits (MDL; ng/mL) detailed in supplementary material
* $p < 0.05$ level of significance, difference in maternal PFAA concentration (ng/mL) of women giving birth to boys or girls

Table 4-4. Distribution of outcomes of the Bayley-III scales of infant development stratified into boys and girls

	Boys (n=252)			Girls (n=237)		
	Mean (SD)	Range	borderline[A]	Mean (SD)	Range	borderline[A]
Cognitive	109 (13)	(90 – 135)	4 (1.6)	113 (14)*	(95 – 145)	0 (0)
Language	108 (14)	(80 – 129)	7 (2.8)	114 (14)*	(86 – 141)	4 (1.7)
Motor	108 (58)	(79 – 127)	8 (3.2)	110 (15)	(82 – 140)	5 (2.1)
Social emotional	105 (13)	(80 – 130)	1 (0.4)	110 (13)*	(85 – 133)	0 (0)
General adaptive	101 (12)	(76 – 121)	9 (4.0)	107 (12)*	(85 – 132)	3 (1.4)

[A] Composite score of the Bayley-III identified as borderline score that was within clinical range, n (%)

* $p < 0.05$ level of significance, comparison of Bayley-III outcomes between boys and girls by student t-test

Table 4-5. Associations of PFAAs and Hg with child outcomes from Bayley-III and an interaction of infant sex (p-value)

	Cognition[A]		**Language**[A]		**Motor**[A]		**Social Emotional**[A]		**General Adaptive**[A]	
	β (95 % CI)	*Sex* [B]	*β (95 % CI)*	*Sex* [B]	*β (95 % CI)*	*Sex* [B]	*β (95 % CI)*	*Sex* [B]	*β (95 % CI)*	*Sex* [B]
PFHpA										
Linear	-0.88 (-1.7, -0.06)*	0.359	0.16 (-0.70, 1.0)	0.989	-0.77 (-1.7, 0.14)	0.421	-0.85 (-1.7, -0.02)*	0.278	-0.79 (-1.6, 0.02)	0.645
Quadratic	0.28 (-0.19, 0.75)		-0.04 (-0.5, 0.46)		0.43 (-0.09, 0.96)		0.41 (-0.07, 0.88)		0.35 (-0.11, 0.81)	
PFOA										
Linear	0.23 (-1.6, 2.0)	0.841	0.16 (-1.74, 2.1)	0.470	-0.31 (-2.7, 2.0)	0.852	0.22 (-1.6, 2.0)	0.361	-0.29 (-2.3, 1.8)	0.667
Quadratic	0.59 (-0.86, 2.1)		-0.002 (-1.5, 1.5)		-0.54 (-2.2, 1.1)		-0.33 (-1.8, 1.1)		-1.1 (-2.5, 0.25)	
PFNA										
Linear	-0.46 (-2.0, 1.1)	0.743	-0.05 (-1.7, 1.6)	0.375	-0.96 (-2.7, 0.78)	0.768	-0.98 (-2.5, 0.57)	0.526	-1.2 (-2.8, 0.38)	0.853
Quadratic	0.50 (-0.27, 1.3)		-0.09 (-0.90, 0.72)		0.47 (-0.37, 1.3)		0.49 (-0.27, 1.3)		-0.31 (-1.2, 0.46)	
PFDA										
Linear	0.43 (-1.2, 2.1)	0.652	0.13 (-1.6, 1.8)	0.309	0.49 (-1.3, 2.3)	0.814	0.52 (-1.1, 2.2)	0.465	-0.65 (-2.3, 0.99)	0.472
Quadratic	0.19 (-0.93, 1.3)		-0.29 (-1.5, 0.88)		-0.78 (-2.0, 0.45)		-0.76 (-1.9, 0.36)		-0.73 (-1.8, 0.35)	
PFUnA										
Linear	-1.5 (-3.5, 0.54)	0.571	-1.5 (-3.6, 0.57)	0.091	-0.78 (-3.0, 1.5)	0.861	0.55 (-1.5, 2.6)	0.135	-0.11 (-2.1, 1.9)	0.086
Quadratic	0.90 (-1.8, 3.6)		-0.61 (-3.4, 2.2)		-1.3 (-4.3, 1.6)		-0.13 (-2.8, 2.5)		-1.4 (-3.9, 1.2)	
PFDoA										
Linear	-2.0 (-3.9, -0.01)*	0.692	-0.59 (-2.6, 1.4)	0.627	-2.1 (-4.2, 0.02)	0.755	0.71 (-1.3, 2.7)	0.116	0.47 (-1.4, 2.4)	0.01*
Quadratic	0.97 (-1.1, 3.1)		-0.72 (-2.9, 1.5)		0.28 (-2.0, 2.6)		-0.73 (-2.8, 1.4)		-1.4 (-3.4, 0.63)	
PFHxS										
Linear	-0.50 (-2.0, 1.0)	0.268	1.0 (-0.58, 2.6)	0.771	0.35 (-1.4, 2.1)	0.098	0.01 (-1.5, 1.5)	0.386	2.2 (0.63, 3.7)**	0.056
Quadratic	0.56 (-0.54, 1.7)		0.21 (-0.94, 1.4)		0.11 (-1.1, 1.3)		-0.35 (-1.5, 0.76)		-0.80 (-1.8, 0.20)	
PFOS										
Linear	1.6 (-0.83, 4.0)	0.877	0.94 (-1.6, 3.4)	0.996	1.0 (-1.9, 4.0)	0.890	0.63 (-1.8, 3.0)	0.533	2.8 (0.17, 5.4)*	0.01*
Quadratic	-2.5 (-6.3, 1.3)		-4.4 (-8.3, -0.43)*		-1.2 (-5.5, 3.0)		1.0 (-2.9, 4.9)		-0.98 (-4.5, 2.5)	
THg[C]										
Linear	-0.76 (-2.2, 0.69)	0.226	-1.1 (-2.6, 0.39)	0.844	-0.13 (-1.7, 1.5)	0.782	-0.30 (-1.7, 1.1)	0.177	-0.76 (-2.2, 0.69)	0.129
Quadratic	0.24 (-0.15, 0.63)		0.4 (0.01, 0.80)*		0.13 (-0.30, 0.56)		0.23 (-0.16, 0.62)		0.24 (-0.15, 0.63)	

Level of significance *p < 0.05, **p < 0.01; [A] Regression models were adjusted covariates significant with neurological assessment outcomes in univariate models; [B] Interaction by infant sex (p-value) assessed by adding interaction term to multivariate models (PFAA x sex);[C] the calculated threshold for the quadratic coefficient (eq. 2) was above the maximum exposure level of Hg (Table 4-2), and discarded from this dataset; β-value represents the change in child score per unit increase in log-transformed PFAA (ng/mL) or Hg (μg/L).

Table 4-6. Associations of PFOS isomers with child outcomes from Bayley-III and interaction of infant sex (p-value)

	Cognition[A]		**Language**[A]		**Motor**[A]		**Social Emotional**[A]		**General Adaptive**[A]	
	β (95 % CI)	*Sex*[B]	*β (95 % CI)*	*Sex*[B]	*β (95 % CI)*	*Sex*[B]	*β (95 % CI)*	*Sex*[B]	*β (95 % CI)*	*Sex*[B]
Linear										
Linear	1.5 (-0.80, 3.8)	0.793	0.49 (-1.9, 2.9)	0.920	1.0 (-1.8, 3.8)	0.930	0.70 (-1.6, 3.0)	0.728	2.7 (0.29, 5.2)*	0.019*
Quadratic	-2.5 (-5.8, 0.79)		-4.0 (-7.5, -0.57)*		-1.0 (-4.7, 2.7)		0.51 (-3.0, 4.0)		-1.6 (-4.3, 1.2)	
∑Branch										
Linear	1.5 (-0.96, 3.9)	0.826	1.8 (-0.73, 4.4)	0.748	0.68 (-2.3, 3.7)	0.765	0.22 (-2.2, 2.7)	0.199	2.1 (-0.55, 4.8)	0.004*
Quadratic	-1.2 (-5.2, 2.8)		-2.7 (-6.9, 1.5)		-0.45 (-4.9, 4.0)		1.8 (-2.1, 5.8)		0.41 (-3.5, 4.3)	
1*m*-										
Linear	-0.37 (-2.3, 1.5)	0.432	0.65 (-1.3, 2.6)	0.829	-0.30 (-2.5, 1.9)	0.745	-0.21 (-2.1, 1.7)	0.287	0.87 (-1.1, 2.8)	0.081
Quadratic	-1.1 (-2.6, 0.42)		-1.8 (-3.3, -0.24)*		-0.35 (-2.0, 1.3)		0.08 (-1.5, 1.7)		-0.32 (-1.8, 1.2)	
∑3*m*+4*m*-										
Linear	2.6 (-0.003, 5.1)	0.969	2.0 (-0.69, 4.7)	0.766	1.9 (-1.2, 4.9)	0.631	0.12 (-2.4, 2.7)	0.416	2.6 (-0.04, 5.3)	0.008*
Quadratic	-0.50 (-4.7, 3.7)		-3.2 (-7.6, 1.1)		-1.1 (-5.7, 3.5)		2.7 (-1.5, 6.8)		-0.50 (-4.5, 3.5)	
5*m*-										
Linear	0.32 (-1.9, 2.5)	0.630	0.72 (-1.6, 3.0)	0.695	-0.28 (-3.0, 2.5)	0.717	-0.12 (-2.3, 2.1)	0.273	1.4 (-1.1, 3.8)	0.010*
Quadratic	0.24 (-2.8, 3.3)		-1.1 (-4.3, 2.1)		0.14 (-3.3, 3.5)		1.3 (-1.8, 4.4)		0.31 (-2.7, 3.3)	
***iso*-**										
Linear	2.3 (-0.16, 4.7)	0.976	2.8 (0.27, 5.3)*	0.775	1.2 (-1.7, 4.1)	0.836	0.43 (-2.0, 2.8)	0.114	2.3 (-0.31, 4.9)	0.005*
Quadratic	-2.3 (-6.1, 1.4)		-3.2 (-7.2, 0.75)		-1.3 (-5.5, 2.9)		1.8 (-1.9, 5.5)		0.33 (-3.3, 4.0)	

Level of significant *p < 0.05, **p < 0.01; [A] Regression models were adjusted covariates significant with neurological assessment outcomes in univariate models; [B] Interaction by infant sex (p-value) assessed by adding interaction term to multivariate models (PFAA x sex)

Table 4-7. Associations of PFAAs and PFOS isomers with Bayley-III scale of general adaptive behaviour stratified into males and females

Male				Female			
PFAAs[A] β (95 % CI)		PFOS Isomers[A] β (95 % CI)		PFAAs[A] β (95 % CI)		PFOS Isomers[A] β (95 % CI)	
PFUnA		**Linear**		**PFUnA**		**Linear**	
Linear	2.1 (-0.72, 4.8)		0.15 (-3.3, 3.6)	Linear	-1.2 (-3.9, 1.5)		4.8 (1.9, 7.8)**
Quadratic	-0.81 (-4.5, 2.8)		-1.8 (-5.9, 2.3)	Quadratic	-2.4 (-5.9, 1.0)		-1.6 (-4.4, 1.2)
PFDoA		**∑Branch**		**PFDoA**		**∑Branch**	
Linear	2.0 (-0.75, 4.7)		-1.3 (-5.0, 2.4)	Linear	-1.3 (-3.9, 1.3)		5.0 (1.6, 8.4)**
Quadratic	-0.63 (-3.8, 2.5)		-0.66 (-5.8, 4.4)	Quadratic	-1.6 (-4.2, 1.0)		0.090 (-5.2, 5.4)
PFHxS		**1*m*-**		**PFHxS**		**1*m*-**	
Linear	0.65 (-1.5, 2.8)		-0.47 (-3.4, 2.4)	Linear	3.4 (1.5, 5.2)**		2.2 (-0.34, 4.6)
Quadratic	-0.41 (-1.9, 1.1)		-0.78 (-2.8, 1.3)	Quadratic	-1.1 (-2.3, 0.03)		0.026 (-2.1, 2.1)
PFOS		**∑3*m*/4*m*-**		**PFOS**		**∑3*m*/4*m*-**	
Linear	-0.34 (-4.0, 3.3)		-0.59 (-4.4, 3.2)	Linear	5.4 (2.2, 8.5)**		5.7 (2.2, 9.1)*
Quadratic	-1.6 (-6.3, 3.1)		-0.25 (-5.3, 4.8)	Quadratic	-1.6 (-5.7, 2.5)		-1.7 (-7.2, 3.7)
		5*m*-				**5*m*-**	
Linear			-1.25 (-4.7, 2.2)	Linear			3.8 (0.78, 6.8)*
Quadratic			-0.83 (-4.9, 3.2)	Quadratic			0.35 (-3.7, 4.4)
		***iso*-**				***iso*-**	
Linear			-1.2 (-4.7, 2.4)	Linear			4.9 (1.6, 8.3)*
Quadratic			-0.55 (-5.5, 4.4)	Quadratic			0.098 (-4.8, 5.0)

Level of significant *p < 0.05, **p < 0.01; [A] Regression models were adjusted covariates significant with neurological assessment outcomes in univariate models

Table 4-8. Association of maternal PFAA exposure and Bayley-III child composite scores including THg co-exposure

	Cognition[A] β (95 % CI)	**Language**[A] β (95 % CI)	**Motor**[A] β (95 % CI)	**Social Emotional**[A] β (95 % CI)	**General Adaptive**[A] β (95 % CI)
PFHpA (*THg)					
Linear	-1.2 (-2.5, -0.01)*	-0.14 (-1.4, 1.1)	-0.90 (-2.3, 0.46)	-1.4 (-2.6, -0.15)*	-0.47 (-1.6, 0.72)
Quadratic	0.24 (-0.24, 0.72)	-0.018 (-0.51, 0.48)	0.41 (-0.12, 0.95)	0.40 (-0.08, 0.88)	0.31 (-0.15, 0.77)
PFNA (*THg)					
Linear	-2.1 (-4.8, 0.49)	-0.83 (-3.5, 1.9)	-3.3 (-6.2, -0.33)*	-3.0 (-5.6, -0.40)*	-1.6 (-4.1, 0.94)
Quadratic	0.58 (-0.19, 1.3)	-0.01 (-0.81, 0.79)	0.59 (-0.26, 1.4)	0.51 (-0.25, 1.3)	-0.32 (-1.1, 0.45)
PFDoA (*THg)					
Linear	-2.3 (-4.9, 0.35)	-1.16 (-3.8, 1.5)	-0.96 (-3.9, 1.9)	0.04 (-2.7, 2.7)	1.4 (-1.2, 3.9)
Quadratic	0.88 (-1.2, 3.0)	-0.89 (-3.1, 1.3)	0.37 (-2.0, 2.7)	-0.74 (-2.9, 1.4)	-1.2 (-3.2, 0.81)
PFHxS (*THg)					
Linear	1.1 (-1.2, 3.4)	2.0 (-0.36, 4.4)	0.85 (-1.8, 3.5)	0.16 (-2.2, 2.5)	2.0 (-0.29, 4.2)
Quadratic	0.51 (-0.61, 1.6)	0.22 (-0.93, 1.4)	0.22 (-1.0, 1.5)	-0.27 (-1.4, 0.87)	-0.69 (-1.7, 0.32)
PFOS (*THg)					
Linear	1.6 (-2.2, 5.5)	-0.63 (-4.6, 3.3)	-1.3 (-5.8, 3.2)	-0.85 (-4.7, 3.0)	-1.0 (-4.8, 2.8)
Quadratic	-2.0 (-5.8, 1.9)	-3.4 (-7.4, 0.54)	-0.46 (-4.8, 3.9)	1.5 (-2.5, 5.5)	-0.08 (-3.6, 3.4)
PFOS Isomers					
Linear (*THg)					
Linear	1.2 (-2.5, 4.8)	-1.3 (-5.0, 2.4)	-1.4 (-5.7, 2.8)	-0.95 (-4.6, 2.7)	-1.1 (-4.7, 2.5)
Quadratic	-2.0 (-5.3, 1.4)	-3.3 (-6.7, 0.19)	-0.48 (-4.3, 3.3)	0.80 (-2.8, 4.3)	-1.3 (-4.1, 1.4)
1*m*- (*THg)					
Linear	1.6 (-1.4, 4.5)	0.75 (-2.3, 3.8)	0.55 (-2.9, 4.0)	0.22 (-2.9, 3.3)	-1.1 (-4.1, 2.0)
Quadratic	-0.94 (-2.4, 0.54)	-1.6 (-3.1, -0.08)*	-0.28 (-1.9, 1.4)	0.09 (-1.5, 1.7)	-0.28 (-1.8, 1.2)
***iso*- (*THg)**					
Linear	2.1 (-1.8, 6.0)	1.1 (-2.9, 5.1)	-1.5 (-4.0, 3.1)	-0.66 (-4.6, 3.3)	-0.86 (-4.8, 3.1)
Quadratic	-2.2 (-6.2, 1.7)	-2.3 (-6.3, 1.7)	-0.30 (-4.7, 4.1)	2.1 (-1.8, 6.0)	1.4 (-2.3, 5.2)

Level of significant *p < 0.05; [A]Regression models were adjusted covariates significant with neurological assessment outcomes in univariate models

Chapter 5

Summary, Conclusions, and Future Directions

5.1 Overview and Current Knowledge Gaps

Currently, knowledge gaps exist in the effects from perfluoroalkyl acid (PFAA) exposure during pregnancy on maternal health and child development, and there is a lack of experimental models investigating neurodevelopment effects from chemical mixtures. To address this, in Chapter 2 I examined co-exposure of PFAAs with methylmercury (MeHg) in a pregnant rodent model and with increasing doses of PFOS. I also utilized questionnaire data and archived biological samples from a Canadian birth cohort (APrON) to: i) examine longitudinal associations of maternal PFAA exposure, and the interaction of mercury (Hg) co-exposure with maternal thyroid hormones in the general population, including a subset of women with high thyroid peroxidase antibodies (TPOAb) (Chapter 3); and ii) investigate associations between maternal PFAA and Hg co-exposure and neurological development in their 2-year-old children (Chapter 4). To date, no previous epidemiological studies of PFAAs and maternal thyroid status or child development have considered isomer-specific associations, and new isomer-specific effects were revealed using my optimized high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS) analysis of perfluorooctane sulfonate (PFOS) isomers.

5.2 Advances in Knowledge

5.2.1. Quantitative Profiling of PFAAs and Their Isomers in Human Samples

Historical (prior to 2002) manufacturing of perfluoroalkyl substances (PFASs) primarily used the Simons Electrochemical Fluorination (ECF) process, resulting in impure mixtures of PFAAs with variable isomeric compositions. Despite the presence of branched isomers in human blood and the environment, chromatographic separation of branched isomers is still an uncommon practice in biomonitoring. Previous methods of isomer quantification were hindered by relatively long sample run times (e.g., 78 min)[1] that are not practical in cohort studies with hundreds, or thousands of participants. To my knowledge, no epidemiological studies investigating the effects of PFAA exposure on thyroid hormone status, or child neurodevelopment, have considered isomer-specific associations prior to this work.

A recent method for quantifying linear and branched isomers of PFAAs in landfill leachate (see Benskin et al.,[2]) was adapted and optimized here for detection and quantification of PFAAs (listed in Table 1-1) and isomers of PFOA and PFOS (listed in Table 1-2 and depicted in Appendix B), in human plasma and serum (see Appendix B). In maternal plasma (n = 429) and serum (n = 65), with the exception of perfluoroheptanoate (PFHpA) (65 %), perfluoroundecanoate (PFUnA) (89 %) and perfluorododecanoate (PFDoA) (56 %), all PFAAs (including PFOS isomers) were detectable in > 99 % of maternal samples; thus, the specialized analytical effort was worthwhile and should be encouraged in future work. Nevertheless, like in prior cohort studies, short-chain PFAAs perfluorobutanoate (PFBA), perfluoropentanoate (PFPA), and perfluorohexanoate (PFHxA) were not detectable, and perfluorooctanoate (PFOA) was primarily observed only as the normal-chain (linear) isomer, with minor amounts of *iso*-PFOA detectable in less than half of samples (45 %), but no other PFOA isomers above detection limits.

One of the practical challenges in birth cohort studies is often the large number of biological samples that must be quantitatively analyzed with specialized instrumentation such as the mass spectrometer, and often these methods prioritize high throughput at the expense of sensitivity and adequate chromatographic separation. In this study, within a sample run time of only 21 min, high sensitivity and excellent chromatographic separation was achieved for quantification of PFAAs ranging from 4 to 14 carbons, and corresponding branched PFOA and PFOS isomers. A fluorinated, pentafluorophenylpropyl (PFP) stationary phase was used over more commonly employed C8 and C18 phases in reverse-phase chromatography, thereby removing endogenous mass interferences (e.g., bile acids), and improving accuracy and sensitivity when quantifying PFAAs in human samples[1].

5.2.2. Developmental Neurotoxicity of Maternal PFAA and MeHg Co-Exposure

In humans, dietary intake and placental transfer of MeHg and PFAAs during pregnancy gives rise to their potential for toxicological interaction that may affect maternal hormone regulation or fetal development. Although PFOS is one of the most prominent PFAAs detected in human blood, no investigations have considered co-exposure of PFOS with MeHg in a developmental toxicity model. To address this, I exposed pregnant rats to either PFOS-alone, MeHg-alone, or a mixture of PFOS (low or high) and MeHg. By comparison to untreated

controls, newborn and juvenile offspring from exposed dams were monitored for achievement of development milestones and simple neurodevelopment tests as newborns, a battery of subsequent developmental tests, and finally the analysis of brain metabolites as juveniles.

Newborn rats from high-mix exposure to PFOS and MeHg showed decreased weight gain and delayed responses in reflex testing from untreated controls that were not observed in either PFOS-only or MeHg-only groups. The results indicated PFOS and MeHg interact, altering activity in early newborn rats. However, in testing the same rats as older juveniles, PFOS-only rats had increased overall activity and MeHg-only rats showed altered behaviour in the open field arena. This was not present in either combined exposure groups, suggestive of an antagonistic interaction. Partial least squares-discriminant analysis (PLS-DA) plots of metabolite profiles in juveniles mirrored the behavioural test results, with significant alterations in the individual chemical exposure groups, but not in either combined exposure groups. Furthermore, altered levels of specific metabolites (e.g., phosphatidylcholines (PCs), γ-amino butyric acid (GABA), and glycine (Gly)) revealed specific target pathways that may be influenced from in utero exposure to PFOS and/or MeHg. Antagonistic effects from co-exposure to PFOS and MeHg are consistent with previous observations of antagonism observed from co-exposure to polychlorinated biphenyls (PCBs) and MeHg[3–5].

This is the first experimental evidence of a toxicological interaction between PFOS and MeHg, that was demonstrated under a highly relevant chronic low-dose exposure during gestation and lactation. My hypothesis that different effects (i.e., on behaviour and metabolite profiles) are elicited from combined exposure to PFOS and MeHg compared to individual chemical exposure was confirmed, but the antagonism was unexpected. One of the strengths of this investigation was the diverse range of metabolites profiled, thereby establishing prospective links between specific cortical metabolites that correspond with altered behaviour. For example, hyperactive behaviour in the open field may be explained by the observed changes in cortical levels of neurotransmitters GABA and Gly. As a preliminary study, this investigation provides a foundation for the potential of chemical mixtures to alter neurochemistry that give rise to changes in behaviour.

5.2.3. Association of Maternal PFAAs and Hg on Thyroid Hormone Status During Gestation and Lactation

Maternal thyroid hormone dysregulation has been linked to adverse child neurodevelopment[6–8]. Past studies of maternal thyroid hormone status have observed associations with increased exposure to PFAAs during pregnancy, but there was inconsistency in the significance of outcomes[9]. Previous epidemiological studies have utilized single sample collections during gestation that may not account for the changes in thyroid hormone levels throughout the duration of pregnancy[10–13].

The study in chapter 3 is one of the first to use a longitudinal analysis and mixed effects statistical approach to identify temporal differences in the associations between PFAAs and thyroid hormones at multiple timepoints throughout pregnancy. PFAAs were analyzed from maternal plasma collected during the 2nd trimester (n = 494), modeled with repeat measures of thyroid hormones during gestation (each trimester) and post-birth (3 months postpartum) to consider changes in their associations with PFAAs. Increasing concentrations of PFHxS and PFOS isomers were positively associated with thyroid stimulating hormone (TSH), but only in the 1st and 2nd trimesters. These findings suggested that associations of PFAAs with TSH were time-dependent, as the strength of these associations changed over the progression of pregnancy. To date, only one other study used repeat measures of thyroid hormones but did not find time-dependent associations, likely due to differences in sample collection time that included one sample during pregnancy (2nd trimester), and other measures post-birth (3 days and 6 weeks)[14]. The overall strength of our study design is from repeat hormone measurements during pregnancy, where PFAAs exhibit the strongest influence in the early stages, a time when fetal neurodevelopment is most susceptible to adverse development.

Maternal thyroid hormone associations with PFHxS were among the most notable. Although most associations of PFAAs and thyroid hormones are discussed in terms of their statistical association, the results of PFHxS with TSH and FT4 provide evidence of a causal relationship when these associations are considered in the context of the Bradford-Hill criteria[15]. PFHxS was the only PFAA to be associated with decreased free thyroxine (FT4), and this was concurrent with increased TSH, observed to occur in a dose-responsive manner (Figs. 4-1 and 4-2), associations that were also present in past cohort studies of PFAA and maternal thyroid

hormones[16–19]. Among all PFAAs examined, PFHxS has the strongest binding affinity for the human thyroid transport protein transthyretin (TTR)[20]. It can be postulated that displacement of T4 from TTR transiently increases serum levels of FT4, but that this stimulates metabolism and excretion, ultimately lowering the amount of bioavailable FT4 at steady-state[21], and through negative feedback stimulates pituitary secretion of TSH (Figure 1-3). This consistency in the literature, biological gradient (dose-response) and significance of associations provide biological plausibility for establishing a causal relationship between PFHxS and maternal thyroid hormone homeostasis during pregnancy[15].

Maternal TSH was significantly associated with branched PFOS isomers but not with corresponding linear-PFOS, despite the linear isomer making up a greater proportion of total-PFOS. As described in chapter 3, PFOS isomers may share physical properties and have molecular sizes that more closely resemble PFHxS. In comparison to linear-PFOS, branched isomers are shorter in chain length and are less hydrophobic, properties that would allow these isomers to interact with biomolecules (e.g., thyroid binding proteins) that exhibit influence over thyroid hormone regulation. These PFOS isomer-specific findings emphasize the importance of isomer-specific analysis and evaluation in future epidemiology studies.

This is the first study to demonstrate the potential for mixture effects from inclusion of total Hg (THg) in statistical models of PFAAs. THg was analyzed in the RBC fraction of whole blood collected in the 2nd trimester. As a predictor variable, increased THg was associated with decreased free triiodothyronine (FT3), and as an interaction term, increased THg with ∑3*m*-+4*m*-PFOS isomers was associated with lowered TSH; an association that was only significant when including the THg interaction. This result suggests that environmental contaminants with common exposure sources may influence the strength of associations for the individual chemicals. Associations were also observed in women categorized as TPOAb positive. PFUnA was negatively associated with FT4, and 1*m*-PFOS was negatively associated with TSH, associations that were not present in women without elevated TPOAb. These results reinforce the "multiple-hit" hypothesis proposed by Webster et al. that thyroid conditions may increase susceptibility to the effects of thyroid disrupting environmental contaminants[18].

During pregnancy, there is increased fetal demand on a maternal supply of thyroid hormones, and iodine deficient women experience pronounced decrease in availability of FT3

and FT4 compared to the marginal decreases of these hormones in iodine sufficient women[22]. Iodine plays central role in thyroid production of T4. Although we considered iodine sufficiency based on intake of prenatal vitamins containing iodine, the lack of measured urinary iodine content in participants is a limitation of this investigation. According to Webster et al. the combination of low iodine and elevated TPOAb represents a vulnerable subgroup within the general US population that are susceptible to PFAA-associated thyroid disruption[23], an outcome that may be of greater concern for studies considering the influence of PFAAs during pregnancy.

5.2.4. Association of prenatal PFAA exposure on child neurological development

Several experimental animal models and human epidemiological studies have demonstrated the potential of PFAAs to act as developmental neurotoxicants. However, only a small number of studies have examined associations of prenatal PFAA exposure on early child development (infancy)[24], and most have solely focused on the influence of PFOA and PFOS, the two most prominent PFAAs detected in human plasma[25,26], ignoring potential associations from other PFAAs. Furthermore, mixture effects from co-exposure to other neurotoxicants with common exposure sources (e.g., Hg) have also not been considered. In this study, PFAAs and THg were analyzed in maternal samples, collected from the same participants in the thyroid hormone investigation (Chapter 3), to examine sex-specific associations with neurological development in two-year-old children (n = 490), assessed using the Bayley Scales of Infant and Toddler Development, 3rd Edition (Bayley-III).

An exploratory analysis of the data prior to modelling indicated non-linear relationships between PFAA exposures and composite scores on Bayley-III subscales. Two prior investigations had also observed non-linear relationships between PFAAs and child development[27] or child incidence of attention deficit hyperactivity disorder (ADHD)[28]. In the current models, a quadratic term was included in regression models to account for these non-linear associations. After adjustment for potential confounders, PFHpA and PFDoA were negatively associated with cognitive scores, and PFHpA was also negatively associated with social-emotional scales of development. Sex-specific associations showed that higher PFHxS and PFOS were positively associated with general adaptive behaviour in girls, whereas boys were unaffected. The non-linear component was only significant for PFOS and its isomers, and thresholds could be estimated for these analytes. Higher total-PFOS was negatively associated

with language scores above a threshold of 5.0 ng/mL, and isomers L-PFOS and 1m-PFOS were negatively associated with language above 3.78, and 1.70 ng/mL respectively.

A Spearman correlation indicated that PFAAs were (generally) not significantly correlated with THg but effect modification of THg on PFAA-neurodevelopment associations was considered. When including THg, PFNA was negatively associated with both motor and social-emotional scales of child development. However, in multi-pollutant investigations, naturally occurring compounds that are not considered in the statistical model may influence the strength of associations. For example, two independent investigations of prenatal MeHg exposure from fish consumption during pregnancy on child neurodevelopment had different outcomes, with MeHg-associated cognitive deficits in children from the Faroe Islands[29] and no evidence in children from the Seychelles[30]. The contrasting results of prenatal MeHg exposure and child development was attributed to long-chain-polyunsaturated fatty acids, whose beneficial properties may mitigate the associated adverse effects from exposure to MeHg[31]. Furthermore, fish also contain the element selenium that has been hypothesized to have a neuroprotective effect, suggesting that dietary selenium delays or mitigates the onset of effects from MeHg[32]. The potential for these naturally occurring compounds to obscure associations of Hg with child development should be considered in future co-exposure investigations but are likely only relevant to populations with increased dietary intake of fish, particularly fish with an increased fatty acid content.

The underlying strength of this study is attributed to the design of the APrON cohort, which allowed for a relatively large sample size of maternal-child pairs, as well as adjustment for numerous potential confounders with information from detailed participant questionnaires. This is one of the first studies of early (i.e., 2-year-old) child development to include PFAA analytes other than PFOS and PFOA, and the first to consider interaction with THg co-exposure. Incorporating a non-linear quadratic term into regression models better fit the data, allowing for more accurate associations to be modeled compared to simple linear regression. It was also demonstrated that the associated effects from PFNA exposure may be potentiated in presence of THg, as associations for PFNA were only significant when considering THg.

PFAA exposure during pregnancy is associated with adverse maternal thyroid hormone status, that was observed to occur in a dose-responsive manner, suggesting that more highly

exposed populations may be at greater risk of PFAA-induced alterations in thyroid hormone status leading to adverse effects on child development. However, it should be mentioned that the PFAAs associated with changes in maternal thyroid hormones (Chapter 3) were different from the PFAAs that were significantly associated with adverse child development (Chapter 4). Although PFAA induced thyroid hormone dysregulation is one of the primarily mechanisms that has been proposed for PFAA-related deficits in neurodevelopment, this was not supported by comparing the results of both investigations.

5.3 Considerations in Statistical Methodology

Within this thesis, careful consideration was given to the statistical approaches used to address each investigation. The animal co-exposure experiment in Chapter 2 had smaller sample sizes and the general output of data followed a non-normal distribution. Thus, non-parametric testing and group comparisons included a Bonferroni correction (see section 2.2.8 for details). Due to practical challenges of conducting animal testing, especially in behaviour studies, experiments are limited by smaller sample sizes, limited statistical power, and a high degree of variability (e.g., rat behavioural responses tend to be inconsistent) and non-significant results discussed in the chapter should be interpreted with a caution. However, significant results and conclusions were determined using 8 replicates per experimental group and a conservative approach (Bonferroni adjustment) allowing for a greater degree of certainty. In Chapters 2 and 3 larger sample sizes were considered in and regression models in the context of a biomonitoring investigation (see sections 3.3.5 and 4.2.5 for details). The availability of participants in the APrON cohort allowed for a relatively high number of participants to be included (approximately 500 participants), resulting in a relatively high statistical power.

5.4 Conclusions of Thesis

Based on the results of the investigations in Chapters 2, 3 and 4, it can be concluded that PFAAs and mercury (i.e., MeHg) can each act as neurotoxicants, and their co-exposure during gestation and lactation results in distinct changes in offspring behaviour and brain chemistry that is different from exposure to either contaminant alone. PFAAs and their isomers were associated with altered maternal thyroid hormones during pregnancy, but such associations are time-dependent and influenced by the presence of external stressors (e.g., THg and thyroid peroxidase

antibodies). Prenatal exposure to PFAAs and their isomers is associated with adverse early child development; as associations of some isomers were stronger (i.e., had increased effect sizes) compared to others. However, there was no evidence to indicate that PFAA-associated thyroid hormone dysregulation is a mechanism of subsequent adverse child neurodevelopment.

5.5. Future Directions

Developmental toxicology studies have traditionally been conducted using increasing doses of single chemicals. In Chapter 2, it was found that developmental exposure to PFOS and Hg can interact, eliciting distinct changes in behaviour and brain chemistry of exposed offspring. Target metabolomics quantified altered levels of specific metabolites using high-resolution mass spectrometry (Orbitrap) that were potentially linked to the observed changes in animal behaviour. Although this included a large set of target metabolites, there may have been effects in the wider metabolome. High resolution mass spectrometry enables for non-target analysis to quantify changes in tens of thousands of metabolites, thereby allowing for unexpected changes to metabolite profiles that are overlooked in targeted metabolomics. From Chapter 2, it was also found that specific metabolites (e.g. PCs, GABA, Gly, etc.,) coincided with observed changes in rat behaviour. Although these observations provide new insight, a detailed mechanism linking exposure to changes in metabolite profiles, and ultimately behavior, remains unclear. A previous study of MeHg and PFOA considered neuronal expression of "immediate early genes" C-Fos and C-Jun, that generally have low expression levels, but whose expression is stimulated from chemical exposure (e.g., MeHg), and are considered markers for induced neuron activity[33]. A follow-up investigation of gene and protein expression using in vitro models of harvested cortical cells would give insight into target pathways affected by single and combined exposures to various environmental contaminants.

Maternal PFAA exposure in other recently published cohort studies was for samples collected in the early 2000s when exposure was known to be higher. For example, investigations of associations of PFAAs with maternal thyroid hormone status[19] and child cognition[27] published in 2018 used data from Project Viva in the USA, with participants enrolled from 1999-2002. Data from this time period was advantageous in its representation of peak human PFAA exposure and thus, may result in stronger associations with PFAAs, but may not be an accurate representation of present-day exposure levels. Moreover, through advancements in technology

and instrumentation, analytical methods have improved sensitivity and precision since the initial quantitation of PFAAs in the early 2000s. Future studies would benefit from more current samples collections that better represent exposure in the present population, and from a more current quantitation method that includes shorter and longer chained PFAAs that have replaced phased-out PFOS and PFOA in recent industrial processes.

In epidemiological studies, isomer-specific effects (i.e., associations) of PFAAs has remained largely unstudied, possibly due to the challenges of chromatographic separation when also weighing the throughput-demands of large population studies. In Chapter 3, branched PFOS was associated with altered thyroid hormone status, whereas the linear isomer of PFOS (and total-PFOS) was not, a finding that is clearly problematic for past or future investigations that only consider total PFOS. Furthermore, branched PFOS has greater transplacental transfer efficiency, suggesting increased fetal exposure to these branched compounds. The method of PFAA quantitation in maternal plasma using a PFP column achieved relatively rapid chromatographic separation of PFAAs and corresponding branched isomers that would benefit future studies. Trends of PFOS in human serum indicate a decreases in most countries, whereas PFHxS has declined more slowly or remained unchanged[34], and observations from recent international birth cohorts have found that North America has some of the highest maternal blood levels of PFHxS (Table 1-3). Its apparent potency to affect maternal thyroid hormone status, its longer half-life, and continued detection in serum suggest that PFHxS may represent the highest risk of adverse effects among all PFAAs today.

Co-exposure to multiple chemicals, or mixtures of contaminants, have generally not been considered in epidemiological studies. The lack of consideration for mixtures may be attributable to the difficulty in adjusting for potential confounders, identifying associations between single or multiple contaminants with specific outcomes, or the occurrence of multi-collinearity as multiple chemicals are sometimes highly correlated and over-represented in statistical models. In Chapter 4, including THg as an interaction in PFAA-neurodevelopment models resulted in changes in these single-chemical associations, suggesting THg increases or decreases the strength of association of PFAAs on child development. Multi-pollutant investigations may use alternative approaches such as PCA, and partial least squares (PLS) regression that offer several advantages over linear regression models, such as minimizing influence of multicollinearity, and considering

that each factor within components of the model represent a weighted contribution of several individual contaminants.

PFAAs and MeHg are ideal candidates for investigating the effects from exposure to contaminant mixtures, sharing common exposure sources, both present in human circulation with relatively long half-lives, and both sharing common targets within the body (e.g., the HPT-axis). Although PFAAs as persistent organic pollutants and Hg as heavy metals make up two major classes of environmental contaminants, they only make up a small proportion of the hundreds of low-level contaminants that humans are routinely exposed to. Future work should include more refined approaches to group contaminants with similar exposure sources and expand our understanding of the exposome, that encompasses the life-course of human exposure to environmental contaminants and their corresponding effects.

5.6 References

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Appendix A – Supporting Information for Chapter 2

A.1 Quality Assurance and Quality Control (QA/QC)

A.1.1 Isomer-Specific PFOS Analysis - Recovery and Precision

Triplicate spike/recovery experiments of PFOS were performed to determine the recovery and precision of PFAA detection and quantitation. The recovery of PFOS was determined from a pre-extraction spiked reference sample, which encompasses both relative recovery and sample matrix effects. PFOS was not detectable in procedure or instrument blanks that were included in QA/QC experiments and was not detected in any equipment or consumables used in sample collection or extraction. Method precision was assessed using the percent relative standard deviation (% RSD) of spike/recovery experiments. Results are presented in Table A-1.

A.1.2 Metabolomic Analysis

The targeted metabolomics method measured 199 metabolites by ultra-high-performance liquid-chromatography tandem mass spectrometry with quantification by authentic standards. Determination of lipids, including acylcarnitines, ceramides, sphingomyelins, phosphatidylcholines, and lyso-phosphatidylcholines was carried out using flow injection tandem-mass spectrometry with isotopic overlap deconvolution together with a limited number of authentic standards. Nomenclature for amino acids utilized a standard 3-letter notation (e.g. alanine = Ala) while lipids were named according to Ribbenstedt et al. Briefly, glycerophospholipids were defined based on the presence of ester and/or ether bonds (represented by an ‘a’ or ‘e’, respectively), a ‘C’ denoting the fatty acid, followed by chain length and the number of double bonds separated by a colon. Two letters (ae = acyl-alkyl, aa = diacyl) denote fatty acids bound to two glycerol positions (e.g., PCaaC20:2). Sphingomyelin nomenclature uses the same nomenclature but with a ‘d’ to denote the backbone sphingosine (e.g. SM (d18:0/C18:1). Carnitines are denoted by a ‘C’ followed by the corresponding number of carbons and double bonds separated by a colon (e.g. C2:0). A complete list of analytes, internal standards, and corresponding nomenclature for metabolomic analysis is found in Table A-2.

Analysis of samples from dissected brain regions of rat offspring were randomized and run with intermittent procedural blanks and sequence quality control samples to minimize instrument carryover and to determine any signal drift. Blank signal subtraction was carried out for compounds detected in the procedural blank. For compounds which showed signs of severe signal drift throughout the sequence, a batch correction algorithm (batchCorr) was applied using the sequence QCs in order to reduce it. Dissected brain regions of hypothalamus, hippocampus, cerebellum, brainstem, and cortex produced distinct metabolomics profiles, a representative PCA plot of brain sections is shown in Figure A-1.

Table A-1. Recovery and precision of triplicate spiked plasma experiments of PFOS linear and branched isomers at a concentration 5 ng/mL of isomer specific (Br-PFOSK) native standards

	L-PFOS	**∑*Br*-PFOS**	***iso*-PFOS**	**5*m*-PFOS**	**∑3*m*+4*m*-PFOS**	**1*m*-PFOS**	**∑*dm*-PFOS**
Product ion (m/z)	499/80 *499/99	-	499/80	499/130	499/130	499/419	499/130
Recovery (%)	129	106	110	78	80	266	-
Precision (% CV)	9	10	17	9	23	25	-

*an additional mass transition was included for linear PFOS

Over-recovery of 1m-PFOS is likely due to the similarity of spiking concentrations to background levels.

Table A-2. List of metabolite class, name, abbreviation and KEGG ID in pathway analysis

Target Class	Target	Abbreviation	KEGG
Amino Acid	Alanine	Ala	C00041
Amino Acid	β-alanine		C00099
Amino Acid	Serine	Ser	C00065
Amino Acid	Homoserine		C00263
Amino Acid	Proline	Pro	C00148
Amino Acid	Valine	Val	C00183
Amino Acid	Alloisoleucine	Aleu	-
Amino Acid	Isoleucine	Ile	C00407
Amino Acid	Leucine	Leu	C00123
Amino Acid	Asparagine	Asn	C00152
Amino Acid	Aspartate	Asp	C00049
Amino Acid	Glutamine	Gln	C00064
Amino Acid	Lysine	Lys	C00047
Amino Acid	Homocysteine		C00155
Amino Acid	Methionine	Met	C00073
Amino Acid	Histidine	His	C00135
Amino Acid	Phenylalanine	Phe	C00079
Amino Acid	Arginine	Arg	C00062
Amino Acid	Threonine	Thr	C00188
Amino Acid	Tyrosine	Tyr	C00082
Amino Acid	Glycine	Gly	C00037
Amino Acid	trans-4-Hydroxyproline		C01157
Amino Acid	Tryptophan	Trp	C00078
Amino Acid	Ornithine		C00077
Amino Acid	Citrulline		C00327
Amino Acid	Glutathione (reduced form)		C00051
Amino Acid/Neurotransmitter	Glutamate	Glu	C00217
Amino Acid (precursor)	3,4-Dihydroxyphenylalanine	DOPA	C00355
BiogenicAmine	Histamine		C00388
BiogenicAmine	Spermidine		C00315
BiogenicAmine/Neurotransmitter	Dopamine		C03758
BiogenicAmine/Neurotransmitter	Serotonin		C00780
Neurotransmitter	GABA		C00334
Neurotransmitter	Thyroxine		C01829
Unclassified	Urea		C00086
Unclassified	Taurine		C00245
Carboxylic acid	Creatinine		C00791
Carboxylic acid	Fumaric Acid		C00122
Carboxylic acid	Succinic Acid		C00042
Carboxylic acid	Malic Acid		C00149
Nucleobase	Adenine		C00147
Nucleobase	Cytosine		C00380

Nucleobase	Uracil		C00106
Nucleobase	Thymine		C00178
Nucleobase	Guanine		C00242
Carnitine	Carnitine	C0	C00487/C00318
Carnitine	L-Acetylcarnitine	C2	C02571
Carnitine	Malonylcarnitine	C2-COOH & C4-OH	
Carnitine	Propionylcarnitine	C3	C03017
Carnitine	Hydroxypropionylcarnitine	C3-OH	
Carnitine	Methylmalonylcarnitine	C3-COOH	
Carnitine	Butyrylcarnitine	C4	C02862
Carnitine	Butenylcarnitine	C4:1	
Carnitine	2-Ethylacryloylcarnitine	C4-Et	
Carnitine	Glutarylcarnitine	C4-COOH	
Carnitine	Valerylcarnitine	C5 (C4-M)	
Carnitine	O-Adipoylcarnitine	C5-COOH	
Carnitine	Hexanoylcarnitine	C6	
Carnitine	2-Hexenoylcarnitine	C6:1	
Carnitine	Heptanoylcarnitine	C7	
Carnitine	Octanoylcarnitine	C8	C02838
Carnitine	Octenoylcarnitine	C8:1	
Carnitine	Nonanoylcarnitine	C9	
Carnitine	O-Sebacoylcarnitine	C9-COOH	
Carnitine	O-Decanoyl-R-carnitine	C10	
Carnitine	Keto-decanoylcarnitine	C10-Ket	
Carnitine	Decenoylcarnitine & Decenoylcarnitine	C10:1	
Carnitine	2-trans,4-cis-Decadienoylcarnitine	C10:2	
Carnitine	Decatrienoylcarnitine	C10:3	
Carnitine		C11 (C9-DM, C10-M)	
Carnitine	Dodecanedioylcarnitine	C11-COOH	
Carnitine	Dodecanoylcarnitine	C12	
Carnitine	Hydroxylauroylcarnitine	C12-OH	
Carnitine	Hydroxydodecenoylcarnitine	C12:1-OH	
Carnitine		C13-COOH	
Carnitine	Tetradecanoylcarnitine	C14	
Carnitine	Hydroxy-tetradecanoyl carnitine	C14-OH	
Carnitine	Tetradecenoylcarnitine	C14:1	
Carnitine	Tetradecadiencarnitine	C14:2	
Carnitine	Hydroxytetradecadiencarnitine	C14:2-OH	
Carnitine	Palmitoylcarnitine	C16	C02990
Carnitine	Palmitoleoylcarnitine	C16:1	
Carnitine	Hydroxyhexadecenoylcarnitine	C16:1-OH	
Carnitine	Hexadecadienoylcarnitine	C16:2	
Carnitine	Hydroxyhexadecadienoylcarnitine	C16:2-OH	
Carnitine	Carboxyheptadecanoylcarnitine	C17-COOH	

Carnitine		C17:1-COOH	
Carnitine	Stearoylcarnitine	C18	
Carnitine	Hydroxyoctadecanoylcarnitine	C18-OH	
Carnitine	Elaidic carnitine	C18:1	
Carnitine	Hydroxyoleoylcarnitine	C18:1-OH	
Carnitine	Hexadecadienoylcarnitine	C18:2	
Carnitine	Hydroxylinoleylcarnitine	C18:2-OH	
Carnitine	Hydroxyoctadecatrienoylcarnitine	C18:3-OH	
Carnitine	Stearidonyl carnitine	C18:4	
Ceramide	N-Butyroyl-D-erythro-sphingosine	Cer(d18:1/4:0)	
Ceramide	N-Hexanoyl-D-erythro-sphingosine	Cer(d18:1/6:0)	
Ceramide	N-Octanoyl-D-erythro-sphingosine	Cer(d18:1/8:0)	
Ceramide	N-(Dodecanoyl)-sphing-4-enine	Cer(d18:1/12:0)	
Ceramide	N-(Tetradecanoyl)-sphing-4-enine	Cer(d18:1/14:0)	
Ceramide	N-(Hexadecanoyl)-sphing-4-enine	Cer(d18:1/16:0)	
Ceramide	N-(Heptadecanoyl)-sphing-4-enine	Cer(d18:1/17:0)	
Ceramide	N-(Octadecanoyl)-sphing-4-enine	Cer(d18:1/18:0)	
Ceramide	N-(9Z-Octadecenoyl)-sphing-4-enine	Cer(d18:1/18:1)	
Ceramide	N-(Nonadecanoyl)-sphing-4-enine	Cer(d18:1/19:0)	
Ceramide	N-(Eicosanoyl)-sphing-4-enine	Cer(d18:1/20:0)	
Ceramide	N-(Docosanoyl)-sphing-4-enine	Cer(d18:1/22:0)	
Ceramide	N-(Tricosanoyl)-sphing-4-enine	Cer(d18:1/23:0)	
Ceramide	N-(Tetracosanoyl)-sphing-4-enine	Cer(d18:1/24:0)	
Ceramide	N-(15Z-tetracosenoyl)-sphing-4-enine	Cer(d18:1/24:1)	
Ceramide	N-(Pentacosanoyl)-sphing-4-enine	Cer(d18:1/25:0)	
Ceramide	N-(Hexacosanoyl)-sphing-4-enine	Cer(d18:1/26:0)	
Ceramide	N-(17Z-Hexacosenoyl)-sphing-4-enine	Cer(d18:1/26:1)	
Phosphatidylcholine		PC aa C20:0	C00157
Phosphatidylcholine		PC aa C24:0	C00157
Phosphatidylcholine		PC aa C26:0	C00157
Phosphatidylcholine		PC aa C28:0	C00157
Phosphatidylcholine		PC aa C28:1	C00157
Phosphatidylcholine		PC aa C28:2	C00157
Phosphatidylcholine		PC aa C30:0	C00157
Phosphatidylcholine		PC aa C30:2	C00157
Phosphatidylcholine		PC aa C32:0	C00157
Phosphatidylcholine		PC aa C32:1	C00157
Phosphatidylcholine		PC aa C32:2	C00157
Phosphatidylcholine		PC aa C32:3	C00157
Phosphatidylcholine		PC aa C34:0	C00157
Phosphatidylcholine		PC aa C34:1	C00157
Phosphatidylcholine		PC aa C34:2	C00157
Phosphatidylcholine		PC aa C38:0	C00157
Phosphatidylcholine		PC aa C38:1	C00157

Phosphatidylcholine	PC aa C38:2	C00157
Phosphatidylcholine	PC aa C38:3	C00157
Phosphatidylcholine	PC aa C38:4	C00157
Phosphatidylcholine	PC aa C38:5	C00157
Phosphatidylcholine	PC aa C40	C00157
Phosphatidylcholine	PC aa C40:1	C00157
Phosphatidylcholine	PC aa C40:2	C00157
Phosphatidylcholine	PC aa C40:3	C00157
Phosphatidylcholine	PC aa C40:4	C00157
Phosphatidylcholine	PC aa C40:5	C00157
Phosphatidylcholine	PC aa C40:6	C00157
Phosphatidylcholine	PC aa C42:0	C00157
Phosphatidylcholine	PC aa C42:1	C00157
Phosphatidylcholine	PC aa C42:2	C00157
Phosphatidylcholine	PC aa C42:4	C00157
Phosphatidylcholine	PC aa C42:5	C00157
Phosphatidylcholine	PC aa C42:6	C00157
Phosphatidylcholine	PC ae C30:0	C05212
Phosphatidylcholine	PC ae C30:1	C05212
Phosphatidylcholine	PC ae C32:1	C05212
Phosphatidylcholine	PC ae C32:2	C05212
Phosphatidylcholine	PC ae C34:1	C05212
Phosphatidylcholine	PC ae C34:2	C05212
Phosphatidylcholine	PC ae C34:3	C05212
Phosphatidylcholine	PC ae C34:4	C05212
Phosphatidylcholine	PC ae C36:0	C05212
Phosphatidylcholine	PC ae C36:1	C05212
Phosphatidylcholine	PC ae C36:2	C05212
Phosphatidylcholine	PC ae C36:3	C05212
Phosphatidylcholine	PC ae C36:4	C05212
Phosphatidylcholine	PC ae C36:5	C05212
Phosphatidylcholine	PC ae C38:0	C05212
Phosphatidylcholine	PC ae C38:1	C05212
Phosphatidylcholine	PC ae C38:2	C05212
Phosphatidylcholine	PC ae C38:3	C05212
Phosphatidylcholine	PC ae C38:4	C05212
Phosphatidylcholine	PC ae C38:5	C05212
Phosphatidylcholine	PC ae C38:6	C05212
Phosphatidylcholine	PC ae C40:1	C05212
Phosphatidylcholine	PC ae C42:0	C05212
Phosphatidylcholine	PC ae C42:1	C05212
Phosphatidylcholine	PC ae C42:2	C05212
Phosphatidylcholine	PC ae C42:3	C05212
Phosphatidylcholine	PC ae C42:4	C05212

Phosphatidylcholine	PC ae C42:5	C05212
Phosphatidylcholine	PC ae C44:3	C05212
Phosphatidylcholine	PC ae C44:4	C05212
Phosphatidylcholine	PC ae C44:5	C05212
Lysophosphatidylcholine	lysoPC a C10:0	C04230
Lysophosphatidylcholine	lysoPC a C12:0	C04230
Lysophosphatidylcholine	lysoPC a C14:0	C04230
Lysophosphatidylcholine	lysoPC a C16:0	C04230
Lysophosphatidylcholine	lysoPC a C17:0	C04230
Lysophosphatidylcholine	lysoPC a C18:0	C04230
Lysophosphatidylcholine	lysoPC a C18:1	C04230
Lysophosphatidylcholine	lysoPC a C18:2	C04230
Lysophosphatidylcholine	lysoPC a C20:0	C04230
Lysophosphatidylcholine	lysoPC a C20:3	C04230
Lysophosphatidylcholine	lysoPC a C20:4	C04230
Lysophosphatidylcholine	lysoPC a C20:5	C04230
Lysophosphatidylcholine	lysoPC a C22:0	C04230
Lysophosphatidylcholine	lysoPC a C22:1	C04230
Lysophosphatidylcholine	lysoPC a C22:5	C04230
Lysophosphatidylcholine	lysoPC a C22:6	C04230
Lysophosphatidylcholine	lysoPC a C24:0	C04230
Lysophosphatidylcholine	lysoPC a C24:1	C04230
Sphingomyelin	SM (d18:0/C16:0)	C00550
Sphingomyelin	SM (d18:0/C16:1)	C00550
Sphingomyelin	SM (d18:0/C18:0)	C00550
Sphingomyelin	SM (d18:0/C18:1)	C00550
Sphingomyelin	SM (OH) (d18:0/C22:2)	C00550
Sphingomyelin	SM (d18:1/C24:0)	C00550
Sphingomyelin	SM (d18:1/C24:1)	C00550
Sphingomyelin	SM (d18:1/C26:0)	C00550
Sphingomyelin	SM (d18:1/C26:1)	C00550

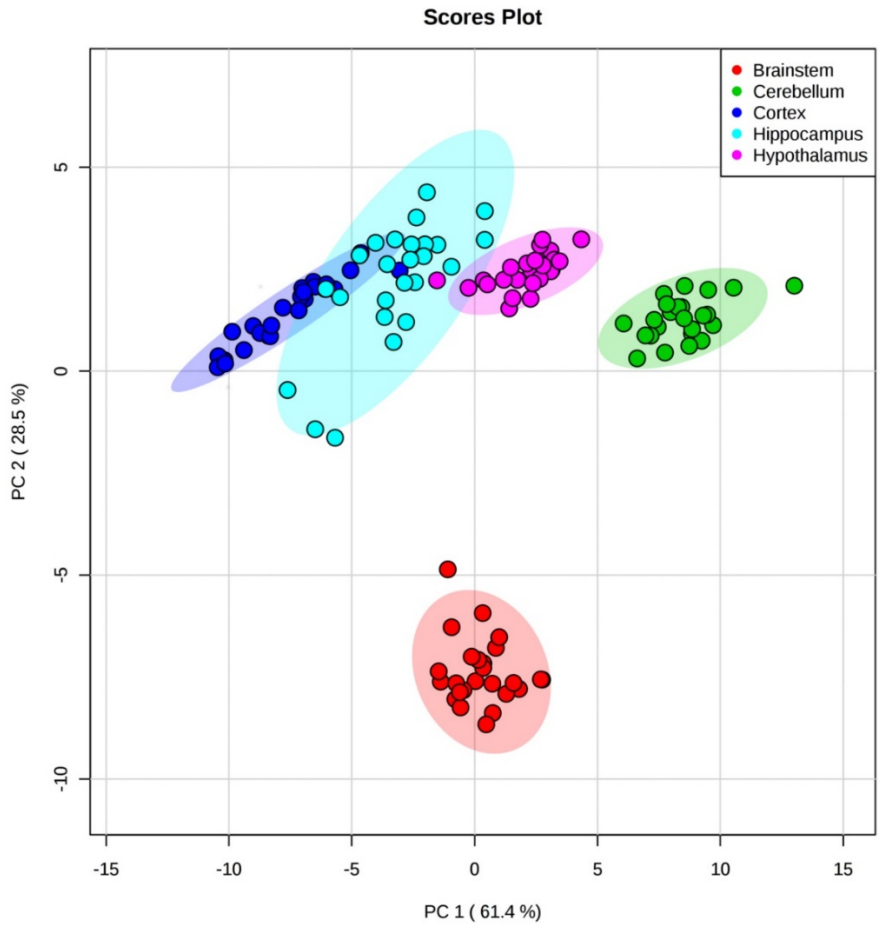

Figure A-1. PCA scores plot of metabolite profiles from all experimental groups of dissected brain regions from Sprague-Dawley rats (n = 25).

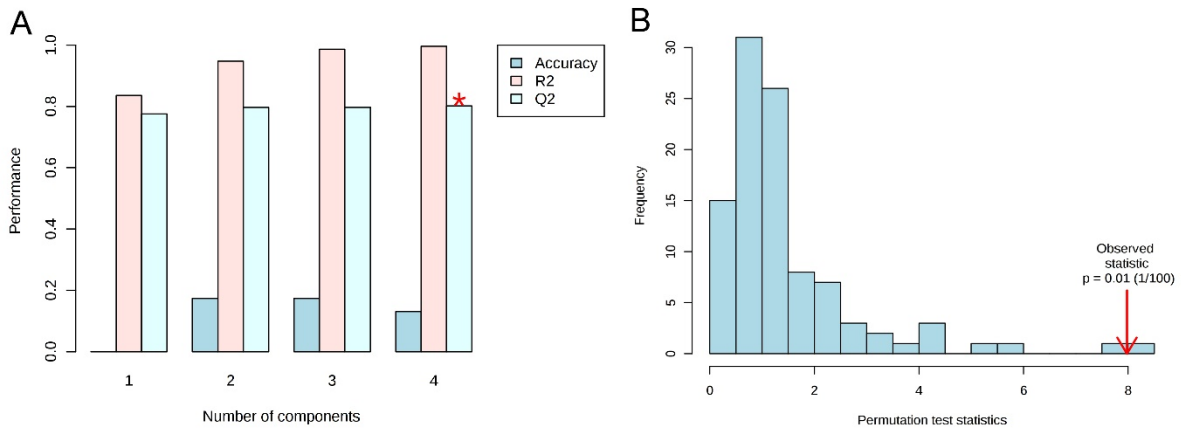

Figure A-2. Validation of PLS-DA model (Figure 8) in cortex section of exposed Sprague-Dawley rat offspring (n = 5), including A) 10-fold cross-validation, and B) permutation testing

Appendix B – Supporting Information for Chapter 3

B.1 Covariate and Sample Collection Selection from Participants of the APrON Cohort

B.1.1 Covariates and Potential Confounders

Covariates were selected based on available demographic data or are known to exhibit a priori influence on thyroid hormones. A summary of selected covariates, including continuous (maternal age) and categorical (education, ethnicity, household income, parity, medical conditions, smoking, alcohol, and recreational drug use) outcomes are shown in Table 3-1. Pre-pregnancy body mass index (BMI) was excluded in our statistical modeling as it has been rationalized that including BMI as a covariate to be adjusted for in models of PFAAs (exposure) and thyroid hormones (outcome) bias statistical models[1,2]. BMI is temporally downstream of PFAAs and thyroid hormones in the causal pathway[3], and adjusting for this "common effect" introduces bias in statistical models[4]. Iodine intake data were obtained through questionnaires, and participants were only included in statistical models if considered iodine sufficient (i.e., taking prenatal supplements during pregnancy that included iodine). A small subgroup of women (n = 25) self-reported to have a diagnosed thyroid condition or taking prescribed medication (e.g. levothyroxine) were included and considered as a potential confounder in mixed effects models.

B.1.2 Sample Collection and Fractionation

Whole blood was collected with either a vacutainer with no additive, left at room temperature for 20 min to coagulate and serum fraction removed; or whole blood collected in a vacutainer containing anticoagulant EDTA, thoroughly mixed by inversion, centrifuged at 3000 rpm for 5 minutes, and plasma faction removed from red blood cell (RBC) fraction. All fractions were labeled and aliquoted into microcentrifuge tubes and stored at -80 ºC until extraction and analysis.

B.2 PFAA Analysis – Quality Assurance and Quality Control

The method of PFAA analysis was based on the original method of Benskin et al.[5], analyzing landfill leachate. Modifications were made to aspects of liquid chromatography (mobile phase composition and gradient elution) and mass spectrometry (instrument parameters

and mass transitions) to optimize conditions for human serum and plasma. Full details of compounds, acronyms, mass transitions and internal standards used in quantitation of PFAA analytes are present in Table B-1.

B.2.1 Reference Samples, Procedure Blanks and Detection Limits

A collection of human plasma was consolidated in a large pooled volume, used throughout the study as an in-house reference standard. An aliquot of reference plasma was extracted and analyzed with each batch of experimental samples. Sample blanks of collection materials and instrument (solvent) blanks did not show any contamination from target PFAA analytes. Procedure blanks were treated in the same manner and run with each batch of experimental plasma samples. The method limit of detection (MDL) was defined as the concentration with a signal to noise ratio of 3 if that PFAA was not detected in the blank. However, small but detectable amounts of contamination from the procedure blanks were observed for PFHpA, linear-PFOS, and linear-PFOA and for these PFAAs, the MDL was defined as the mean concentration plus 3x the standard deviation of procedure blanks. Method detection limits and proportion of PFAAs detected in the overall sample population are present in Table B-2. Analytes detected in > 50 % of the overall population were considered in statistical analyses.

B.2.2 PFAA Recovery and Precision

Triplicate spike/recovery experiments were performed by spiking pre-extracted reference plasma samples with linear (MPFAC-MXB) and isomer specific (Br-PFOSK, and T-PFOA) native standards and internal standard. Recovery encompasses both relative recovery and matrix effects, first using the response (target peak area/IS peak area) to calculate the concentration and subtract the value of non-spiked reference plasma, then calculate recovery by dividing by the corresponding concentration value in standards prepared in MeOH × 100%. Method precision was assessed using the percent relative standard deviation (% RSD) of spike/recovery experiments. Results are presented in Table B-3.

B.3 Mercury Analysis – Quality Assurance and Quality Control

Calibrators were prepared by serial dilutions, which were run to determine the method linearity. All calibrators were prepared a factor of 50 times higher than the final concentrations required. For example, in order to inject 5µg/L on the instrument, 250µg/L calibrator should be prepared as it will be diluted with the basic diluents. The instrumental LOD and LOQ were determined from the linearity data. LOD and LOQ are the concentrations which have at least 3x the absolute abundance in the blank solution, i.e., Cal 1. The reporting limit is the LOQ multiplied by 50. The linearity, LOD, LOQ, and reporting limit for mercury are presented in Table B-4. For the recovery experiment, blank RBC samples and 2 different spike levels were prepared and injected 10 times and the results are present in Table B-5. The calibration standards Seronorm™ whole blood trace elements level 1 and level 2 were used to determine accuracy and precision. Each QC sample was run 6 times on 3 different days. Within-run and between-run precision are tabulated in Table B-6.

B.4. Main Effect Associations and Interaction of PFAAs and Thyroid Hormones

Overall main effect associations of maternal exposure to PFAAs and thyroid hormone status are present in Table B-7. Effect size of interaction terms and level of significance (p-values) are also listed for the interaction of time, birth, THg co-exposure, and TPOAb status when main effect associations were statistically significant ($p < 0.05$).

B.5 The Interaction of TPOAb

Considering of TPOAb as a categorical interaction using a threshold to indicate a subpopulation of women with elevated TPOAb (> 9 mIU/mL) that were more susceptible to PFAA exposure than women with normal TPOAb (< 9 mIU/mL). The adjusted effect of PFAA exposure was reported separately for both groups. The normal TPOAb group was reported as the adjusted effect of PFAA, whereas the elevated TPOAb was reported as the adjusted effect of PFAA including the interaction of TPOAb.

B.6 References

(1) Webster, G. M.; Venners, S. a; Mattman, A.; Martin, J. W. Associations between

Perfluoroalkyl Acids (PFASs) and Maternal Thyroid Hormones in Early Pregnancy: A Population-Based Cohort Study. *Environ. Res.* **2014**, *133C*, 338–347.

(2) Webster, G. M.; Rauch, S. A.; Marie, N. S.; Mattman, A.; Lanphear, B. P.; Venners, S. A. Cross-Sectional Associations of Serum Perfluoroalkyl Acids and Thyroid Hormones in U.S. Adults: Variation According to TPOAb and Iodine Status (NHANES 2007-2008). *Environ. Health Perspect.* **2016**, *124* (7), 935–943.

(3) Pearce, E. N. Thyroid Hormone and Obesity. *Curr. Opin. Endocrinol.* **2012**, *19* (5), 408–413.

(4) Cole, S. R.; Platt, R. W.; Schisterman, E. F.; Chu, H.; Westreich, D.; Poole, C. Illustrating Bias Due to Conditioning on a Collider. *Int. J. Epidemiol.* **2010**, *39*, 417–420.

(5) Benskin, J. P.; Ikonomou, M. G.; Woudneh, M. B.; Cosgrove, J. R. Rapid Characterization of Perfluoralkyl Carboxylate, Sulfonate, and Sulfonamide Isomers by High-Performance Liquid Chromatography-Tandem Mass Spectrometry. *J. Chromatogr. A* **2012**, *1247*, 165–170.

Table B-1. Instrument parameters of PFAAs examined in the present study, including compound, acronym, precursor and product ions, and internal standards used for quantitation

Compound	Acronym	Precursor Ion	Product Ion	Internal Standard
Perfluoroalkyl Carboxylates				
Perfluorobutanoate	PFBA	213	169	13C-PFBA
Perfluoropentanoate	PFPeA	263	219	13C-PFHxA
Perfluorohexanoate	PFHxA	313	269	13C-PFHxA
Perfluoroheptanoate	PFHpA	363	169	13C-PFOA
		363	319	13C-PFOA
Linear-Perfluorooctanoate	linear-PFOA	413	369	13C-PFOA
6m-Perfluorooctanoate	6m-PFOA	413	169	13C-PFOA
5m-Perfluorooctanoate	5m-PFOA	413	219	13C-PFOA
4m-Perfluorooctanoate	4m-PFOA	413	119	13C-PFOA
3m-Perfluorooctanoate	3m-PFOA	413	169	13C-PFOA
∑dm-Perfluorooctanoate	∑dm-PFOA	413	169	13C-PFOA
Perfluorononanoate	PFNA	463	419	13C-PFNA
		463	219	13C-PFNA
Perfluorodecanoate	PFDA	513	469	13C-PFDA
		513	219	13C-PFDA
Perfluoroundecanoate	PFUnA	563	519	13C-PFUnA
		563	219	13C-PFUnA
Perfluorododecanoate	PFDoA	613	569	13C-PFDoA
		613	169	13C-PFDoA
Perfluorotridecanoate	PFTrA	663	619	13C-PFDoA
		663	169	13C-PFDoA
Perfluorotetradecanoate	PFTeA	713	669	13C-PFDoA
		713	169	13C-PFDoA
Perfluoroalkyl Sulfonates				
Perfluorobutane Sulfonate	PFBS	299	80	13C-PFOS
		299	99	13C-PFOS
Perfluorohexane Sulfonate	PFHxS	399	80	13C-PFOS
		399	99	13C-PFOS
		399	119	13C-PFOS
Linear-Perfluorooctane Sulfonate	linear-PFOS	499	80	13C-PFOS
		499	99	13C-PFOS
6m-Perfluorooctane Sulfonate	6m-PFOS	499	80	13C-PFOS
5m-Perfluorooctane Sulfonate	5m-PFOS	499	130	13C-PFOS
∑4m+3m-Perfluorooctane Sulfonate	∑4m+3m-PFOS	499	130	13C-PFOS
1m-Perfluorooctane Sulfonate	1m-PFOS	499	419	13C-PFOS
∑dm-Perfluoroactane Sulfonate	∑dm-PFOS	499	130	13C-PFOS
Perfluorodecane Sulfonate	PFDS	599	80	13C-PFOS
		599	99	13C-PFOS
Internal standards				
	13C-PFBA	217	172	-
	13C-PFHxA	315	270	-
	13C-PFOA	417	372	-
	13C-PFNA	468	423	-
	13C-PFDA	515	470	-
	13C-PFUnA	565	520	-
	13C-PFDoA	615	570	-
	13C-PFOS	503	80	-

Table B-2. Median, standard deviation, arithmetic and geometric means, range and detection limits of PFAAs in maternal plasma (n=494)

PFAA	AM ng/mL	GM (SD) ng/mL	MDL ng/mL	n (%) > MDL[A]
Perfluoroalkyl carboxylates				
PFBA	-	-	0.02	0 (0)
PFPeA	-	-	0.04	0 (0)
PFHxA	-	-	0.02	1 (0.2)
PFHpA	0.14	0.040 (0.33)	0.02	329 (66.6)
Linear-PFOA	2.88	2.12 (3.37)	0.37	494 (100)
6m-PFOA	0.081	0.07 (0.03)	0.01	225 (45.5)
5m-PFOA	0.038	0.03 (0.01)	0.03	17 (3.44)
4m-PFOA	0.039	0.03 (0.01)	0.03	8 (1.62)
3m-PFOA	-	-	0.10	0 (0)
∑dm-PFOA	-	-	-	0 (0)
PFNA	1.05	0.76 (1.27)	0.06	489 (98.9)
PFDA	0.38	0.26 (0.51)	0.04	493 (99.8)
PFUnA	0.21	0.16 (0.15)	0.03	437 (88.5)
PFDoA	0.051	0.03 (0.05)	0.02	274 (55.5)
PFTrA	0.051	0.02 (0.04)	0.03	31 (6.30)
PFTeA	0.021	0.01 (0.01)	0.02	18 (3.64)
Perfluoroalkyl sulfonates				
PFBS	-	-	0.4	0 (0)
PFHxS	1.52	1.01 (1.76)	0.03	494 (100)
linear-PFOS	2.72	2.34 (1.49)	0.12	494 (100)
6m-PFOS	0.50	0.43 (0.28)	0.01	494 (100)
5m-PFOS	0.38	0.32 (0.23)	0.03	494 (100)
∑3m+4m-PFOS	0.26	0.23 (0.23)	0.03	494 (100)
1m-PFOS	0.08	0.06 (0.05)	0.01	478 (96.8)
∑dm-PFOS	-	-	-	0 (0)
PFDS	-	-	-	0 (0)

Abbreviations: AM (arithmetic mean), GM (geometric mean), SD (standard deviation), MQL (method quantitation limit)

[A] number and percentage from sample population below method detection limits (MDL; ng/mL) described above

- reported value is below method detection limit

Table B-3. Recovery and precision of triplicate spike plasma experiments of PFAA linear and isomer analytes, spiked at a concentration 5 ng/mL of linear (MPFAC-MXB), and isomer specific (Br-PFOSK, and T-PFOA) native standards

PFAA	**Recovery** (%)	**Precision** (% RSD)
PFBA	137	19
PFPeA	141	15
PFHxA	117	12
PFHpA	105	2
Linear-PFOA	100	11
Iso-PFOA	116	18
5*m*-PFOA	106	16
4*m*-PFOA	104	12
3*m*-PFOA	135	37
∑*dm*-PFOA	-	-
PFNA	99	12
PFDA	111	12
PFUnA	142	23
PFDoA	105	10
PFTrA	75	27
PFTeA	52	28
PFBS	117	7
PFHxS	103	5
Linear-PFOS	104	10
Iso-PFOS	229	20
5*m*-PFOS	79	28
∑3*m*+4*m*-PFOS	182	7
1*m*-PFOS	182	7
∑*dm*-PFOS	-	-
PFDS	88	17

Table B-4. Linearity, LOD and LOQ of mercury obtained from calibrators prepared in serial dilutions

	Concentration (μg/L)		
	Linearity range	LOD and LOQ	Reporting Limit
^{202}Hg (μg/L)	0 - 2	0.005	0.25

Table B-5. Recovery of mercury in prepared spiked red blood cell samples

	Low spike level[a,b] (0.1 µg/L)		High spike level[a] (1 µg/L)	
	% Recovery	**% CV**	**% Recovery**	**% CV**
^{202}Hg (µg/L)	63.71	8.09	92.35	1.64

[a]Measurements were repeated for 10 injections at each of the low and high spike levels

[b]Recovery at the low spike level failed due to a high background (~25 ppb) in the blank red blood cells

Table B-6. Accuracy and precision of mercury, using Seronorm™ Whole Blood Trace Elements

Seronorm™ Trace Elements Whole Blood L-1 (Ref 210105, Lot#1103128)

		Within-run						Between-run	
	target	Run 1		Run 2		Run 3			
	(µg/L)	% CV	% from target	% CV	% from target	% CV	% from target	% CV	% from target
^{202}Hg	16	4	-1	2.8	-4.5	3.4	-4.6	3.6	-3.4

Table B-7. Overall main effect of PFAAs on thyroid hormones, and outcome from models including interaction of time, pregnancy, THg, and TPOAb in mixed effects models

		Main Effect [A]	**Interactions**			
			Time [A B]	**Birth** [A C]	**THg** [D]	**TPOAb** [E]
		β (p-value)	β (p-value)	β (p-value)	β (p-value)	β (p-value)
TSH	PFNA	0.005 (0.810)	-	-	-	-
	PFDA	0.023 (0.647)	-	-	-	-
	PFUnA	-0.024 (0.901)	-	-	-	-
	PFOA	0.007 (0.368)	-	-	-	-
	PFHxS	0.144 (0.008)	-0.051 (0.014)	0.108 (0.001)	-	-
	total-PFOS	0.082 (0.069)	-	-	-	-
	linear-PFOS	0.005 (0.792)	-	0.057 (0.0476)	-	-
	∑Br-PFOS	0.286 (0.038)	-0.107 (0.045)	0.262 (0.002)	-	-
	1*m*-PFOS	0.379 (0.396)	-	-	-	-2.79 (0.008)
	∑3*m*+4*m*-PFOS	0.026 (0.888)	-	0.67 (0.022)	-0.301 (0.047)	-
	5*m*-PFOS	0.851 (0.028)	-0.311 (0.038)	0.801 (0.001)	-	-
	iso-PFOS	0.053 (0.585)	-	0.315 (0.036)	--	-
	THg	0.019 (0.359)	-	-	-	-
FT4	PFNA	-0.004(0.379)	-	0.011 (0.032)	-	-
	PFDA	-0.010 (0.314)	-	0.026 (0.037)	-	-
	PFUnA	-0.037 (0.321)	-	-	-	-0.230 (0.049)
	PFOA	-0.002 (0.138)	-	0.004 (0.034	-	-
	PFHxS	-0.006 (0.034)	-	-	-	-
	total-PFOS	-4.82E-06 (0.999)			-	-
	linear-PFOS	0.002 (0.656)	-	-	-	-
	∑Br-PFOS	-0.002 (0.810)	-	-	-	-
	1*m*-PFOS	-0.116 (0.198)	-	-	-	-
	∑3*m*+4*m*-PFOS	0.019 (0.59)	-	-	-	-
	5*m*-PFOS	-0.012 (0.565)	-	-	-	-
	iso-PFOS	0.001 (0.965)	-	-	-	-
	THg	0.005 (0.203)	-	-0.011 (0.032)	-	-
FT3	PFNA	0.004 (0.330)	-	-	-	-
	PFDA	0.003 (0.736)	-	-	-	-
	PFUnA	0.014 (0.698)	-	-	-	-
	PFOA	0.001 (0.535)	-	-	-	-
	PFHxS	0.0002 (0.955)	-	-	-	-
	total-PFOS	-0.0028 (0.242)			-	-
	linear-PFOS	-0.004 (0.248)	-	-	-	-
	∑Br-PFOS	-0.008 (0.284)	-	-	-	-
	1*m*-PFOS	-0.122 (0.188)	-	0.454 (0.0001)	-	-
	∑3*m*+4*m*-PFOS	-0.036 (0.290)	-	-	-	-
	5*m*-PFOS	-0.028 (0.182)	-	-	-	-
	iso-PFOS	-0.011 (0.533)	-	-	-	-
	THg	-0.016 (7.33E-05)	-	-	-	-

[A] Effect of PFAAs on thyroid hormones after adjustment of significant covariates from univariate models
[B] Includes time as an interaction term, the change between visits (over all Timepoints)
[C] Includes birth as an interaction term, the change from during pregnancy to post-pregnancy (Timepoint 4 only)
[D] Includes Hg (change with Hg concentration) and [E] TPOAb (change with levels of TPOAb) as interaction terms
- missing values for interaction terms, excluded when not statistically significant ($p < 0.05$)

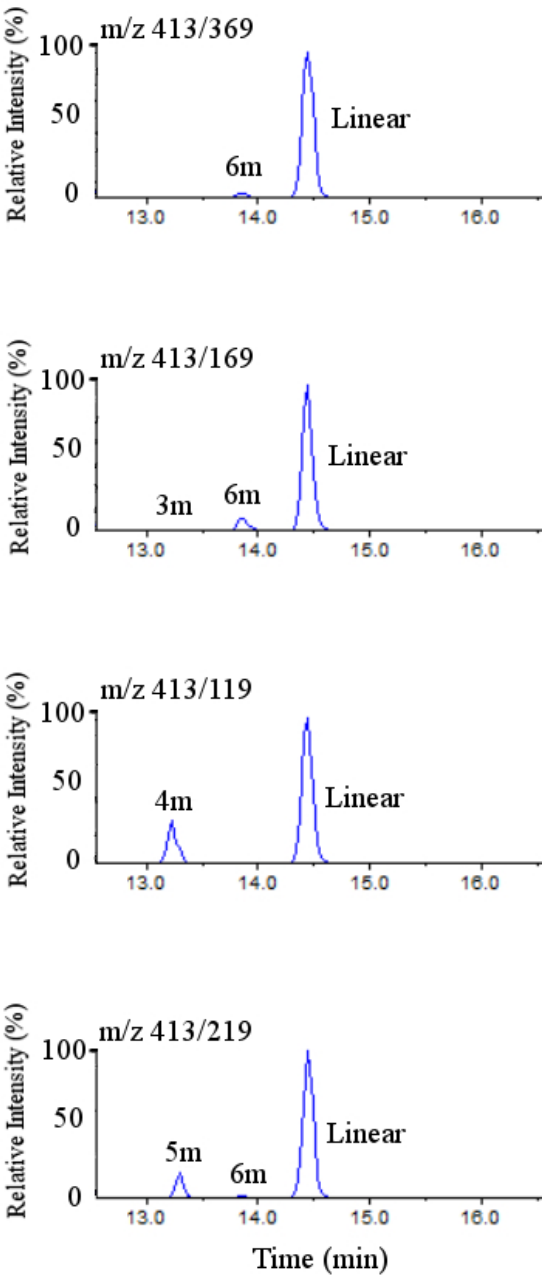

Figure B-1. Chromatogram of PFOA isomer profiles from standards, including mass transitions and relative intensity

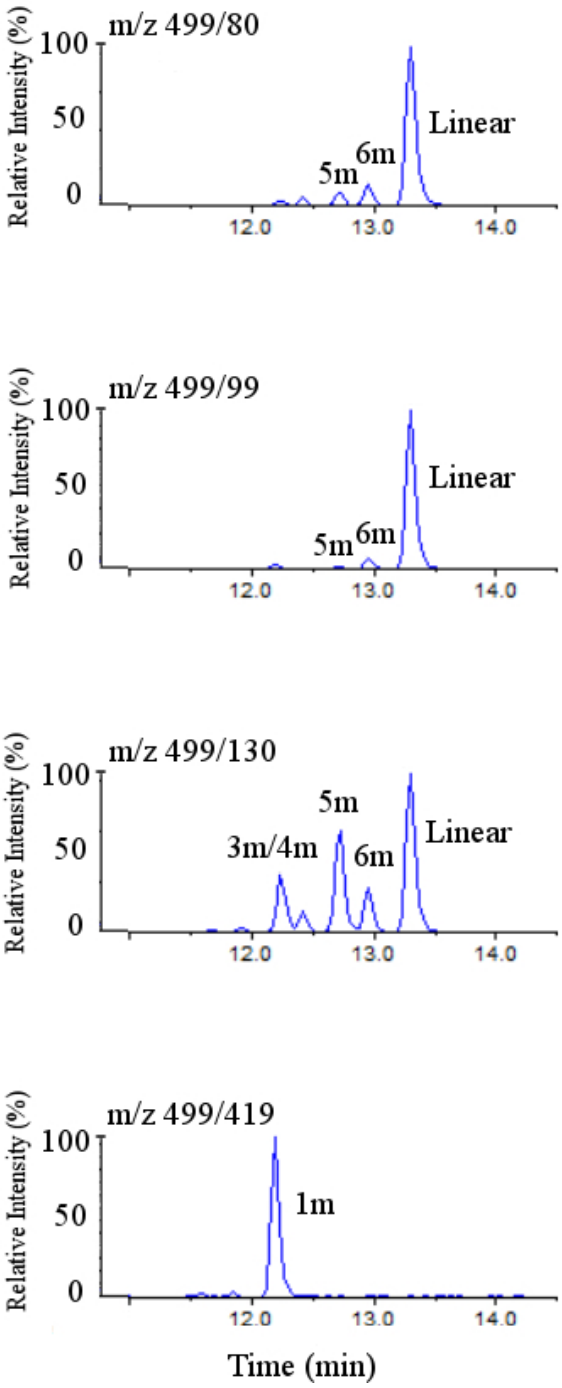

Figure B-2. Chromatogram of PFOS isomer profiles from standards, including mass transitions and relative intensity

Appendix C – Supporting Information for Chapter 4

C.1 Maternal PFAA and Hg Exposure

Full details of instrument parameters and QA/QC data for the complete sample population considered in this thesis (n = 501) are present in Appendix B. Due to missing maternal questionnaire data, a subset of participants was considered for the neurodevelopment study (n = 490). A Spearman correlation of this subset identified perfluoroalkyl carboxylates to be highly correlated with each other and weakly correlated with PFOS; PFHxS was moderately correlated with PFOS, and not with any carboxylates (Table C-1). THg was moderately correlated with PFUnA only.

Table C-1. Spearman Correlation of PFAAs, THg, branched PFOS isomers measured in maternal plasma (n = 490)

	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFHxS	PFOS	THg
PFHpA	-								
PFOA	.487*	-							
PFNA	.443*	.802*	-						
PFDA	.459*	.765*	.813*	-					
PFUnA	.219*	.346*	.471*	.511*	-				
PFDoA	.267*	.480*	.451*	.478*	.265*	-			
PFHxS	.123	.317*	.111	.030	.050	.030	-		
PFOS	.093	.309*	.208*	.152*	.183*	.177*	.456*	-	
THg	-.040	-.040	-.030	-.020	.410*	.050	-.070	.060	-

Isomers of PFOS

	linear-PFOS	1*m*-PFOS	∑3*m*+4*m*-PFOS	5*m*-PFOS	*iso*-PFOS
linear	-				
1*m*-	.580*	-			
∑3*m*+4*m*-	.849*	.611*	-		
5*m*-	.791*	.727*	.898*	-	
iso-	.874*	.582*	.930*	.930*	-

*level of significance, $p < 0.05$