University of Alberta

Human Exposure Sources and Disposition of Perfluoroalkyl Acid Isomers

by

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"You can't connect the dots looking forward; you can only connect them looking backwards. So you have to trust that the dots will somehow connect in your future…"

Steve Jobs

(Feb 24, 1955 – Oct 5, 2012)

Abstract

Perfluoroalkyl acids (PFAAs) are a family of emerging toxicants that are non-biodegradable, and have the potential to bioconcentrate and biomagnify. In an attempt to answer questions about human exposure to PFAAs, and subsequent human disposition of these compounds which have public health implications, four hypothesis-driven and multidisciplinary research projects were conducted. The first project used an isomer-specific LC-MS/MS method to characterize the PFAA isomer signatures in house dust, and whether PFAA isomers could cross the human placenta to different extents. The findings showed that a Canadian population was currently exposed to a mix of both telomer and electrochemical perfluorooctanoate (PFOA), despite the latter having been phased out since 2002. Strong evidence for the preferential transfer of branched perfluorooctanesulfonate (PFOS) and PFOA isomers, compared to linear, was found by comparing 20-paired maternal and cord blood samples. The second project was conducted on a local family of seven individuals, all of whom had exceptionally high serum levels of perfluorohexanesulfonate (PFHxS). A forensic investigation was conducted in the family's dwelling, and data revealed that the source of the high exposure in this family was the house carpets which had been treated multiple times with Scotchgard™ formulations over a period of 15 years. The pathway of exposure was most-likely through inhalation and ingestion of house dust. A series of in vitro experiments were conducted in a third project to assess the dissociation constants (K_d) of linear and branched PFOA and PFOA isomers. K_d's of the linear isomers were lower than the branched isomers, and in technical mixtures spiked to serum it was evident that the branched isomers were less bound to serum protein, and thus would be more bioavailable to cross into fetal circulation or be excreted by the kidney. The last research project modeled the transplacental transfer of PFAAs in-vitro using cultured human placental syncytiotrophoblasts. This model confirmed the findings of the first study, to the effect that branched isomers, especially in the case of PFOA, crossed the placental barrier more efficiently than linear molecules,

independent of their serum protein binding. Overall, this thesis contributed unique observations from biomonitoring of PFAAs at the isomer-specific level, and follow-up experiments helped to explain these while furthermore contributing to our understanding of how PFAAs are biopersistent in humans.

Preface

This thesis represents a series of manuscripts which have been published, or are in preparation for submission to peer-reviewed journals. Thus, duplication of some sections (introduction, materials, methods, etc.) was inevitable. All manuscripts and chapters were written by Sanjay Beesoon with critical comments and edits provided by Jonathan W. Martin. Contributions of all co-authors are provided in detail below.

Chapter One - Sources and pathways of human exposure to perfluoroalkyl acids and their relevance to human health

A review paper is in preparation with Sanjay Beesoon and Jonathan Martin as authors and will be submitted for peer-review.

Chapter Two - Isomer profiles of perfluorochemicals in matched maternal, cord and house dust samples; manufacturing sources and transplacental transfer.

Published in – Environmental Health Perspectives: **(2011)** 119(11):1659-1664.

Author List – Sanjay Beesoon ,Glenys Webster, Mahiba Shoeib, Tom Harner , Jonathan P Benskin , Jonathan W Martin.

Contributions – Sanjay Beesoon conducted sample analysis, data collection, interpretation, and manuscript preparation. Glenys Webster was responsible for participant recruitment and sample collection. Mahiba Shoeib and Tom Harner provided the sieved dust. Jonathan Benskin provided useful suggestions on the

isomer specific analysis. This work was conducted under the guidance of Jonathan Martin.

Chapter Three - Exceptionally high serum concentrations of perfluorohexane sulfonate in a Canadian family linked to home carpet treatment applications.

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Author List – Sanjay Beesoon, Stephen J. Genuis, Jonathan P Benskin, Jonathan W Martin

Contributions - Sanjay Beesoon designed the project, collected the samples, conducted sample analysis, data collection, interpretation, and manuscript preparation. Stephen J. Genuis was the first clinical contact and recruited the participants. Jonathan Benskin provided useful suggestions on the isomer specific analysis and editorial comments. This work was conducted under the guidance of Jonathan Martin.

Chapter Four - Relative binding affinities of PFOS and PFOA isomers for serum proteins.

A manuscript will be submitted for publication in a peer-review journal.

Author List – Sanjay Beesoon, Jonathan W. Martin

Contributions - Sanjay Beesoon designed the project, collected the samples, conducted sample analysis, data collection, interpretation, and manuscript preparation. This work was conducted under the guidance of Jonathan Martin.

Chapter Five - Transfer of PFOS and PFOA isomers across an in-vitro model of the human placental barrier.

A manuscript will be submitted for publication in a peer-review journal.

Author List - Sanjay Beesoon, Joey Sheff, Ashley Davey, Denise Hemmings, Jonathan Martin and Jonathan W. Martin

Contributions – Sanjay Beesoon conceptualized the experiment, conducted sample analysis, data collection, interpretation, and manuscript preparation. Joey Sheff helped with biotransformation experiments as a summer undergraduate student. Ashley Davey harvested the cytotrophoblasts cells from human placenta. Denise Hemmings gave her expert advice on cell culture and provided the laboratory facilities in the Department of Obstetrics and Gynecology. This work was conducted under the guidance of Jonathan Martin and Denise Hemmings.

Chapter Six - Summary, conclusions and future work.

Excerpts will be published as part of the Review Paper in Chapter 1.

Author List – Sanjay Beesoon and Jonathan Martin

Contributions – Sanjay Beesoon prepared this section with editorial comments by Jonathan W. Martin.

Related Published Manuscripts

1. Martin JW, Asher BJ, **Beesoon** S, Benskin JP, Ross MS. *PFOS or PreFOS?* **2010.** Are perfluorooctane sulfonate precursors (PreFOS) important determinants of human and environmental perfluorooctanesulfonate (PFOS) exposure? *Journal of Environmental Monitoring.* **2010** 12:1979-2004.

2. Wang Y, **Beesoon S**, Benskin JP, De Silva AO, Genuis SJ, Martin JW. **2011**. Enantiomer Fractions of Chiral Perfluorooctanesulfonate (PFOS) in Human Sera. *Environmental Science & Technology* 45:8907-8914.

3. Zhang Y, Beesoon S, Zhu L, Martin JW. **2013**. Biomonitoring of perfluoroalkyl acids in human urine and estimates of biological half-life. *Environmental Science & Technology* 47:10619–10627.

4. Zhang Y, **Beesoon S**, Zhu L, Martin JW. **2013**. Isomer-Specific Perfluoroalkyl Acid Analysis in Human Serum from Two Cities in North China. *Environment International*. 53: 9-17

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There was no way I could have done this work without the unconditional support and love of my wife, Mridula, and kids (Shweta, Yashna and Harsh), who sacrificed so much while I was studying; usually parents sacrifice for their kids and not the other way around - I can never pay you back for what you did. I can't forget my brothers (Deepak and Ramesh) and sisters (Indira, Soomantee and Louloune) back home who have always helped me to steer in the right direction during rough times since my early childhood. My thoughts also go to my late Mom (Madoduth) and Dad (Dayanand), who never gave up on their kids' education in spite of dire poverty; your hard work, integrity and humility have inspired us all to be resilient in face of adversity.

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1.1 Chemistry of Perfluorinated Organic Substances

Fluorine is the most electronegative element in the periodic table and is a pale yellow gas at room temperature and pressure. First isolated by the French Chemist Henri Moissan in 1886 by an electrolytic method, the latter is still the method of choice for its industrial production nowadays. When chemically bound to other elements, fluorine can exist as either inorganic or organic compounds. The main industrial uses of inorganic fluorine compounds have been in steel making and aluminum refining. The other important use of inorganic fluoride ions is in the drinking water supply, where there is convincing epidemiological evidence that it offers protection against dental caries in children *(Armfield et al. 2013).* Contrary to inorganic fluorine chemicals which are abundant in the natural environment, organic fluorine compounds are almost always man-made, with the exception of some volcanic and geo-thermal emissions, such as fluoroalkanes *(Gribble 2002)* and monofluoroacetic acid in some rare plants in South Africa and Australia (*Twigg et al. 1996*).

In simple terms, an organic fluorine compound is defined as one containing the elements carbon and fluorine. Thus compounds containing at least one carbon-fluorine bond may be represented by thousands of different compounds of widely varying molecular sizes, elemental composition, and chemical and physical

properties. Given the electronegativity difference on the Pauling scale between carbon and fluorine (2.5 versus 4.0) (*Sen and Jorgenson 1987)*, the C-F bond is extremely polar and is the single strongest covalent bond in organic chemistry (125 kcal/mol) *(Douvris and Ozerov 2008; Jongwook et al. 2011)*. Therefore, it is not surprising that *per*fluorinated compounds (i.e. organofluorine compounds wherein all C-H bonds are replaced by C-F) have exceptional chemical and physical properties such as high thermal stabilities, and resistance to attack by other chemicals making them remarkably inert. Furthermore, the small dipole moments of organic fluorine compounds *(Barry 2008)* and the low critical surface energies of $-CF_2$ and $-CF_3$ moieties *(Audenaert et al. 1999)* contribute to the low friction coefficients of perfluorinated compounds, and also to their unique hydrophobicities and lipophobicities.

1.2 Historical Development of Perfluoroalkyl Substances

In a brilliant review article entitled "Perspective on Fluorocarbon Chemistry" Lemal *(2004)* described perfluorinated compounds as having "**fluorine skins covering carbon skeletons**" with unique properties which make them ideal candidates for a whole range of applications. The discovery of polytetrafluoroethylene (PTFE) by pure serendipity by Roy Plunkett *(Chemical Heritage Foundation 2010; Plunkett 1941)* in 1938 was a turning point in the science of organofluorine chemistry. Shortly after its discovery, the first immediate use of PTFE was in the Manhattan Project during the Second World War. Actually, the US Department of Defense was in dire need for chemically inert material in the equipment to separate Uranium-235 from Uranium-238 by

differential diffusion of UF_6. Enriched uranium was later used to make the atomic bomb *(Leblanc 2006)*. Although the US patent, with little information disclosed, was awarded to Roy Plunkett through Kinetic Chemicals Inc. *(Plunkett 1941)* in 1941, it was not until 1946 that the real first civilian use of PTFE was made by the Dupont company under the trade name Teflon®. In 1948, a patent was issued to Joseph Simons *(Simons 1949)*, then working for the Minnesota, Mining and Manufacturing (3M) Company for his novel electrochemical fluorination (ECF) method of producing fluorocarbons and other organofluorine compounds. Although the 3M Company started organo-fluorine based stain repellents under the trade name Scotchgard™ in 1956, the patent for these repellents came in 1973 *(Patsy and Samuel 1967)*. Given the unique properties of these compounds, the good press they were getting in the 1950's, and more importantly the approval of Teflon® cookware and Zonyl® products in food packaging by the U.S Food and Drug Administration increased consumer confidence and familiarity in these perfluorinated chemical products, and the market for these specialty chemicals thus increased over the decades.

Given the inertness and stability of organofluorine compounds, there has been extensive research and development into their potential applications in the biomedical sciences. For example, fluorocarbons have been tested for use in ophthalmology clinics as artificial vitreous replacements *(Kobuch et al. 2001; Meinert et al. 1985; Soman and Banerjee 2003; Sparrow et al. 1992; Versura et al. 2001)*, as inhalation anesthetics *(Chilcoat et al. 1985; Cuignet et al. 2002; Ragaller et al. 2000)*, as blood substitutes for transfusion (*Chang 2003; Flaim*

1994 ; Geyer 1988; Habler et al. 2005; Kim and Greenburg 2004; Li et al. 2011; Lowe 2003; Yin et al. 2011), and as pulmonary drug delivery agents (*Lehmler 2007*). As a matter of fact, the introduction of fluorine on a candidate drug can significantly improve its pharmaceutical properties such as improved duration of action (*Hagmann 2008*), enhanced central nervous system penetration (*Mahar Doan et al. 2002*), superior potency (*Barnard et al. 1993)* and attenuation of biliary clearance *(Hagmann 2008).* Not surprisingly, of all the drugs licensed worldwide for the past 50 years, 5-15 % has been organofluorine compounds *(Hagmann 2008*).

Perfluoroalkyl substances (PFASs) are defined as compounds where all the carbons (except the carbon associated with the functional group) are fully fluorinated. Examples of PFASs are aliphatic perfluorocarbons (PFCs), perfluoroalkylacids (PFAAs), perfluoroalkane sulfonyl fluorides (POSF), perfluoroalkane sulfonamides (PFOSA), perfluoroalkyl iodides and perfluoroalkyl aldehydes. In this thesis the focus will be on the perfluoroalkyl substances, specifically the PFAAs.

1.3 Perfluoroalkylacids (PFAAs)

PFAAs have the general formula $C_nH_{2n+1}X$, where *n* is typically in the range of 4 to 14, and X is a functional acid group such as a carboxylic ($-COO^-$), sulfonic ($-SO_3^-$) or phosphonic ($P(=O)(OH)_2$) acid moiety, termed perfluoroalkyl carboxylates (PFCAs), perfluoroalkyl sulfonates (PFSAs), and perfluoroalkyl phosphonates (PFPAs), respectively. These chemicals are unique in the sense that they are both hydrophobic and lipophobic, which in turn make them excellent

candidates as surfactants in both regular consumer products and industrial processes. The extensive use of these fluorinated surfactants over the past 5 decades is due to their unparalleled performance at lower concentrations compared to hydrocarbon surfactants. This excellent performance is in turn attributable to four key properties *(Buck et al. 2012)* of the PFAAs, namely:

1. They can substantially decrease aqueous surface tension and are effective at extremely low concentrations (parts per million). They can be used in both acidic and basic aqueous media as well as in organic solvents.
2. Their wetting, spreading and levelling properties are outstanding, no matter on what kind of surface (hard, wet, irregular, plastic, metal, porous or wood) they are applied to.
3. They are excellent emulsifiers, one example being in the production of fluoropolymers.
4. They have exceptional chemical and thermal stabilities.

1.4 Manufacturing Methods

In general, there are 2 main methods for manufacturing perfluoroalkyl substances on an industrial scale. The first one, electrochemical fluorination (ECF), was discovered by Simons et al. in the 1930's, but published later in 1949 as a series of five papers *(Simons et al. 1949*). ECF was the predominant manufacturing method for PFAAs from 1950-2000. Briefly, this process involves the electrolysis of a hydrocarbon sulfonyl fluoride [$H(CH_2)_nSO_2F$ where n=4,6,8,10)] in a solution of hydrogen fluoride (HF) and leads to all the hydrogen atoms on the carbon backbone, and on the functional group, being replaced by fluorine,

yielding an acid fluoride $F(CF_2)_nSO_2F$. This latter compound is the most important raw material from which different types of fluorinated surfactants can be produced as shown in Figure 1.1 below (*adapted from Buck et al. (2012)*). Given the conditions of the electrochemical cell, the likelihood of carbon chain fragmentation and rearrangement is quite high, resulting in a mixture of linear, branched, cyclic and even some trace levels of other impurities such as shorter chain-length PFCAs and PFSAs. For example, the percentage ratio of linear to branched isomers of perfluorooctane sulfonate (PFOS) is 70:30 and for perfluorooctanoate (PFOA) is 80:20.

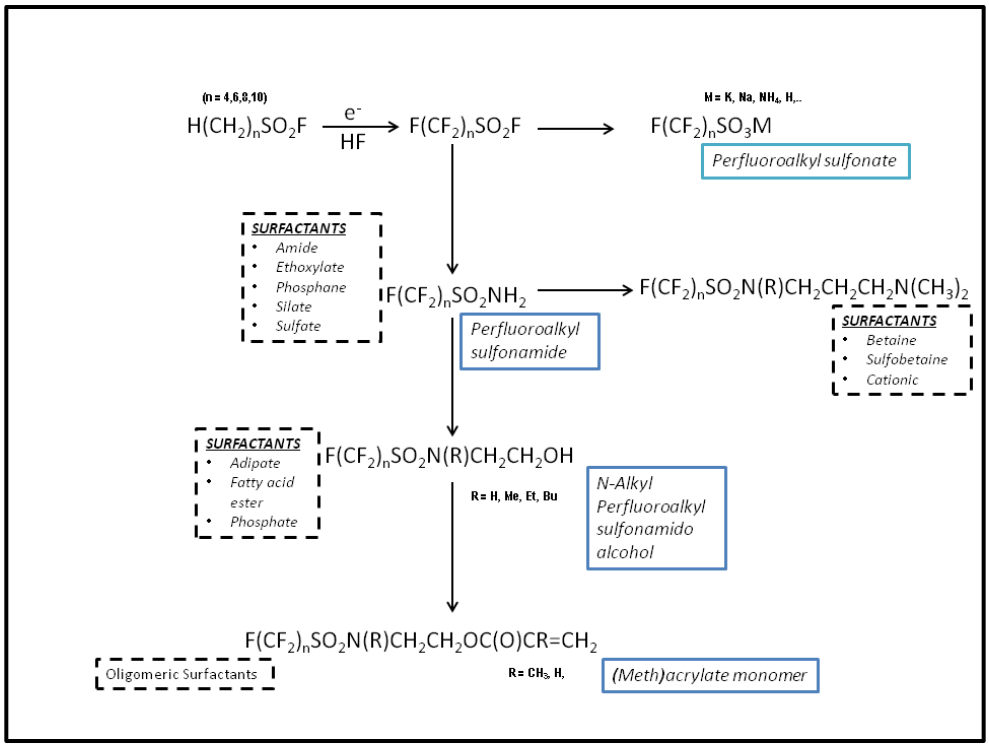


Figure 1.1 Electrochemical fluorination for the production of perfluorooctane sulfonyl fluoride (POSF), which is then used in the manufacture of various surfactants. PFCAs were also manufactured by the 3M Company to make PFCAs. (Figure adapted from Buck et al. 2012)

The alternative method of PFAA production which has become increasingly used, after the 3M Company ceased manufacturing of perfluorooctanesulfonate (PFOS) and perfluorooctanoic acid (PFOA) by ECF, is telomerization *(Banks et al. 1994; Kissa 1994)*. By contrast to ECF, this is a well ordered and controlled reaction which adds -C_2F_4- groups sequentially on a carbon backbone chain. The starting reagent is tetrafluoroethylene which is then iodinated in the presence of a palladium as catalyst to yield pentafluoroiodoethane (see equation 1 below). To this latter product, tetrafluoroethylene is then added at a ratio that produces a target compound of a desired carbon chain length (equation 2). The final step involves oxidation of the perfluoroalkyliodide to yield the perfluorocarboxylic acid of the desired chain length. *(Hekster et al. 2003; Buck et al. 2012).* The end products of telomerization are therefore almost exclusively linear molecules, and usually have even-numbered carbon chain-lengths. In some instances the starting material, pentafluoroiodoethane, is replaced by heptafluoroisopropyl iodide (C_3F_7I), which can lead to branched, and odd-chain length, telomer perfluoroalkyl carboxylates.

$$5C_2F_4 + IF_5 + 2I_2 \xrightarrow{\text{Catalyst}} 5C_2F_5I \qquad (1)$$

$$C_2F_5I + nC_2F_4 \longrightarrow C_2F_5(C_2F_4)_nI \qquad (2)$$

$$C_2F_5(C_2F_4)_nI \xrightarrow{\text{COOH}} C_2F_5(C_2F_4)_{n-1}CF_2COOH \qquad (3)$$

1.5 Uses

Given the exceptional properties of PFAAs, their widespread use in regular consumer products as well as in industrial applications is not surprising. Although

a detailed review of the uses of individual PFAAs is beyond the scope of this thesis, a brief overview of their commercial applications is given next.

1.5.1 Aqueous Film Forming Foams (AFFF)

Given unique wetting, spreading and surface tension lowering properties of PFSAs and their derivatives, they have been used extensively in AFFFs in fire-fighting. When jet fuels and other organic solvents catch fire, the AFFF prevents fire from spreading out by forming a thin film over the fuel (*Cortina 2009)* which cuts off the supply of oxygen. Not surprisingly, PFOS and other PFAAs have been detected in ground water and in river water close to airports and other air-force military facilities *(Moody and Field 2000; Moody et al. 2001; Moody et al. 2002).* The perfluorinated surfactants used in AFFF can be anions, cations or non-ionic.

1.5.2 Coating Applications

The outstanding wettability, levelling properties, stain resistance, corrosion resistance, stability in extreme temperatures and anti-static properties *(Dewitte et al. 1995; Barry 2008; Dams 2009)* make PFAAs ideal candidates for coating a whole range of consumer products such as carpets, textiles, mattresses and upholstery. They have also been incorporated in paints as they substantially improve the surface appearance, give better flow and leveling, and enhanced oil repellency (*Meng 2007*).

1.5.3 Cleaning Agents

Both anionic and cationic fluorinated surfactants have been incorporated in cleaning fluids as they are very efficient at removing dirt from all sorts of hard surfaces (wood, glass, countertops and flooring), and also at providing a protective layer on the surface, thus reducing the likelihood of dirt sticking to that surface. (*Leach 1998)*

1.5.4 Metal Plating

Being both thermally and chemically stable in hot chromic acid, PFAAs were extensively used in electrolytic baths to deposit chromium on both metals and plastics (*Buck et al. 2012*). In the electrolytic process of metal plating, the fluorinated surfactants, in particular PFOS, form stable foams and which are excellent acid mist suppressants *(Brooke 2004)*. Furthermore, given their exceptional stabilities and their efficiencies at lowering surface tension, PFAAs are used in the electrowinning of copper (*Annich and Sierakowski 1995).*

1.5.5 Electronics and semiconductor materials.

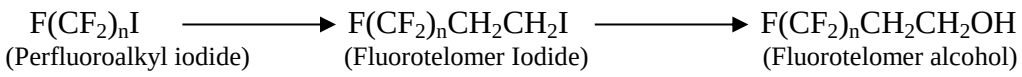
PFOS has multiple uses in the electronics industry and is important at various steps in the production processes for both electric and electronic parts. Photolithography, which is an optical means for transferring patterns onto a substrate, is one of the critical steps in semiconductor manufacturing as it defines the sophistication level and performance of electronic devices. PFOS is used on photoresist materials, which allow etching of images smaller than the wavelengths of light onto silicon wafers and anti-reflective coatings, which prevent the formation of blurred images produced by surface reflections *(European*

Semiconductor Industry Association 2013). A study by *Lin et al. (2009)* found high concentrations of PFOS, PFOA and perfluorodecanoate (PFDA) in river water samples collected downstream from major semiconductor and electronics industries in Taiwan. Despite much research and development, an alternative for PFOS in the electronics industry has not yet been found, and the Stockholm Convention on Persistent Organic Pollutants still allow Parties of the Convention to produce and use PFOS in the electronics industry *(De Baere 2011; Wang et al. 2009*).

1.5.6 Paper and Packaging Industry

With unique properties for repelling both oil and water, PFAAs and related products are used in approximately 8% of the total worldwide paper market (*United Nations Industrial Development Organization 2012*), especially in the food industry where grease repellence is highly desirable. The paper protectors marketed by the 3M Company to be used in food packaging (such as plates, cups, food containers, bags and wrap) and other commercial applications can be classified in two broad categories based on their chemistries. The first category includes mixtures of mono- (10%), di- (85%) and tri- (5%) phosphate esters of N-ethyl perfluorooctanesulfonamidoethanol (N-EtFOSE). The second category is a co-polymer containing N-methyl perfluorooctane sulfonamidoethyl ester (N-MeFOSEA). Compounds falling under these two categories were regulated by the U.S Food and Drug Administration (FDA) for direct food contact under Food Contact Substances (FCS) Notification of the Code of Federal Regulations (CFR) Title 21. Thus, it was not surprising that in recent years quite a few studies have

shown the presence of multiple PFAAs, and fluorotelomer alcohols (FTOHs) which are contemporary replacements of N-EtFOSE and N-MeFOSEA, and their derivatives in food contact paper products such as microwave popcorn bags *(Sinclair et al. 2007; Trier et al. 2011b)* and disposable drinking cups and plates *(Trier et al. 2011a)* and their migration into food *(Begley et al. 2005).*



FTOHs are also manufactured by telomerization (section 1.4) in a reaction sequence as shown above *(Buck et al. 2012)*

1.5.7 Enhanced Oil Recovery and Mining (EORM)

To accommodate the increasing global oil demand (*Gruenspecht 2011),* Government agencies around the world are pushing oil companies to develop enhanced oil recovery technologies to extract the maximum possible of the original-oil-in-place for every site. In that context the use of perfluorinated surfactants have proved to be very effective based on: (1) enhancing foam stabilities, (2) superior subterranean wetting, and (3) modification of surface properties of the reservoir to avoid water blocking of the wellbore (*Murphy and Hewat 2008).*

1.5.8 Photographic industry

One of the major problems faced by photographic film manufacturers is the accumulation of electrostatic charge on the surface of the film during production (especially when surfactants such as gelatin or hydrocarbons are used), transport

in cameras, and during photofinishing *(Orem and Andrews 2004)*. PFOS and PFOS-based chemicals have proved to be very efficient at controlling static electricity on the surface of photographic films, and also at decreasing friction, repelling dirt and preventing adhesion for both digital and analogue imaging films. *(Dams et al. 1996; United Nations Industrial Development Organization 2012).*

1.5.9 Polymerization Aid

Over the past decades, a major direct use for PFAAs has been as polymerization agents in the synthesis of fluoropolymers. The most notable example is the use of PFOA as polymerising agent in the coating of Teflon® cookware with polytetrafluoroethylene (PTFE). *(Prevedouros et al. 2006*)

1.5.10 Pesticides

N-ethyl perfluorooctane sulfonamide (N-EtFOSA) is another compound related to the PFAA family that has proved enormously useful in the agricultural sector, as they are very effective against termites, cockroaches and ants. Although banned in many Western countries, it is still licensed for use in the three major agricultural economies in the world, namely Brazil, China and India. In addition to the direct use of N-EtFOSA as an insecticide, other polyfluorinated chemicals are used as enhancers (inert surfactants) in other pesticide products *(United Nations Industrial Development Organization 2012).*

1.6 Environmental Distribution of PFAAs

With long historical usage in a wide variety of products and industries, and the fact that PFAAs are non-biodegradable, they can now be detected in the abiotic environment, and in organisms. The first report by *Giesy and Kannan (2001)* of the global presence of these compounds in wildlife catalysed a whole series of biomonitoring studies across the world looking at the levels of these compounds in human populations, marine and terrestrial animals, oceans, rivers, soil, sediments, snow caps, indoor and outdoor air and dust samples. These are reviewed briefly below.

1.6.1 Wildlife

Because PFAAs are not very volatile and were not expected to travel long distances in the atmosphere, it was rather surprising when reports came out on the presence of these compounds in remote wildlife (*Giesy and Kannan. 2001*). Other examples are the presence of long chain PFAAs in polar bears, arctic fox, ringed seal, lake whitefish and lake trout in the Canadian Arctic (*Martin et al. 2004*), polar bears, birds and fish from East Greenland and the Faroe Islands (*Bossi et al. 2005*), pandas in China *(Dai et al. 2006)*, birds from the Baltic Sea (*Holmström and Berger 2008*) and penguins from Antarctica (*Schiavone et al. 2009)*. How these chemicals move from industrialized regions to such remote global locations, and what the contributions of semi-volatile PFAA-precursor chemicals are, to the body burden of PFAAs in wildlife at these locations is still subject to scientific debate *(Armitage et al. 2006; Wania F. 2007; Young et al. 2007; Armitage et al.*

2009; Dreyer et al. 2009; Nash et al. 2010; Franco et al. 2011; Del Vento et al. 2012; Zhao et al. 2012; Kwok et al. 2013).

The use of the concepts and models of fugacity to explain long range transport and accumulation of some persistent organic compounds in remote locations was first proposed by Donald Mackay in 1979 *(Mackay 1979)* in a paper entitled "Finding fugacity feasible". Since then, multiple studies *(Ditoro et al. 1991; Bidleman 1999, Wania et al. 2006)* have used the fugacity based approach to predict the movement of semi-volatile persistent organic chemicals in the atmosphere. However, given that PFAAs (for example PFOS and PFOA) are non-volatile at normal temperature, pressure, and at natural aquatic pH, it is a challenge to apply fugacity models to explain their long range transport. However, fugacity models can still be applied to volatile precursors of PFAAs, such as fluorotelomer alcohols, as recently published by *Xiao et al. (2012).* To characterize the source of PFOA and other long chain perfluorocarboxylates (PFCAs) in the Arctic Ocean, *Wania (2007),* used simulation models which incorporate emission estimates and other chemistry parmeters such as fugacity, K_{OW} and vapour pressures to distinguish between oceanic and atmospheric transport routes. It was concluded that, for PFOA, slow ocean transport was likely the dominant pathway to the Arctic Ocean.

1.6.2 Ocean Water

In a research paper "A global survey of perfluorinated acids in oceans" *Yamashita et al. (2005)* reported on the presence of PFOA, PFOS, PFNA and PFHxS at picogram per litre levels in ocean water samples from the Pacific and Atlantic

Oceans, and furthermore described the seasonal and spatial variability in the concentrations. Ahrens et al. (*Ahrens et al. 2009; Ahrens et al. 2010)* have also extensively published on the presence of PFAAs in Northern European and South Atlantic Ocean waters, while Benskin et al. *(Benskin et al. 2012a; Benskin et al. 2012b*) used isomer specific analytical tools to characterize these chemicals in the Arctic and Atlantic oceans to track their manufacturing sources.

1.6.3 River Water

PFAAs have also been extensively studied in rivers from around the world and there is general consensus across studies that PFAA levels in water samples are linked to their proximity to industrial or populated sites. PFAAs have been detected in river water in various countries: United States *(Benskin et al. 2010; Delinsky et al. 2010; Nakayama et al. 2007; Nakayama et al. 2010; Sinclair et al. 2006),* Brazil *(Quinete et al. 2009),* Germany *(Becker et al. 2008; Möller et al. 2010; Wilhelm et al. 2008),* Italy *(Loos et al. 2007),* China *(So et al. 2007),* India (*Yeung et al*. 2009) and Japan *(Saito et al. 2004).* The presence of PFAAs in river water represents both a direct exposure pathway for humans through the drinking water supply and also indirectly through contaminated fish from rivers.

1.6.4 House Dust

Of relevance to human exposure to PFAAs is the consistent presence of these chemicals in household dust. Dozens of studies have documented the presence of the most common PFAAs, namely PFOS, PFOA and PFHxS in house dust at ng/g and in some case at μg/g levels in dust from homes in the United States *(Kato et al. 2009; Knobeloch et al. 2012; Strynar and Lindstrom 2005; Strynar and*

Lindstrom 2008), Canada *(Beesoon et al. 2011; Kubwabo et al. 2005),* Sweden *(Awasum Bjorklund et al. 2009),* Norway *(Haug et al. 2011),* UK *(Harrad et al. 2010),* Belgium (*D'Hollander et al. 2010),* Australia *(Goosey and Harrad 2011),* China *(Zhang et al. 2010)* and Japan *(Moriwaki et al. 2003).* Isomer–specific characterization is dust has been reported only once *(Beesoon et al. 2011).* Inhalation and ingestion of contaminated house dust is probably the most important pathway of PFAA intake for toddlers and young children in high exposure scenarios as suggested by *Trudel et al. (2008a)* in a theoretical predictive model, and by *(Beesoon et al. (2012)* for a highly exposed Canadian family (Chapter 3).

1.6.5 Indoor Air

Inhalation of contaminated air and suspended particles in air is another non-negligible pathway through which humans can be exposed to PFAAs or to their precursors. The most common detectable precursors of PFCAs and PFSAs are the fluorotelomer alcohols (FTOHs) (*Wallington et al. 2006*) and the perfluoroalkyl sulfonamidoethanols (FOSEs) respectively. Their presence in air samples from around the world *(Barber et al. 2007; Berger et al. 2005; Cai et al. 2012; Chaemfa et al. 2010; Dreyer et al. 2009a; Dreyer et al. 2009b; Eisenreich et al. 1981; Goosey and Harrad 2012; Harada et al. 2005; Jahnke et al. 2007; Langer et al. 2010; Martin et al. 2002; Shoeib et al. 2004; Shoeib et al. 2006; Stock et al. 2005)* confirms the ubiquity of these chemicals in the environment.

1.6.6 Food

Food is also a major source of PFAS in human populations. Multiple studies have probed into the levels of PFAS in common food items consumed by the general population in different countries. Among the most notable studies, are those from *Tittlemier et al. (2005;2006;2007)* which consistently showed significantly high levels (ng/g) of PFOS, long chain PFCAs and PFOSA in solid food composite samples collected in Canada. Furthermore, based on PFAS levels in 36 composite food samples purchased in Catalonia (Spain) and in the blood of 48 residents of that area, *Ericson et al. (2007;2008)* reported a correlation between human blood levels of PFOS and dietary intake, with the major contributors being fish, dairy products and meat. In an effort to compare PFOS and PFOA intake through either food or dust inhalation, Zhang et al. (2010*)* analysed food of animal-origin and dust samples from various regions in China and concluded that over 99% of PFOS and 98 % of PFOA total daily intakes are from food. A major study, by *Fromme et al. (2007)* analyzed 214 duplicate diet portions collected from 15 females and 16 males in Munich, Germany, and found median PFOS and PFOA concentrations of 0.025 ng/g and 0.05 ng/g respectively. Another important study conducted in the U.K *(Clarke et al. 2010)* on 252 distinct food samples collected in 2007 and 2008, including vegetables, fruits, canned food, seafood and meat, found PFOS to be the most important PFAS, followed by PFOSA (highest concentration in whitebait) and with PFOA ranking third in importance.

1.7 Human Biomonitoring Studies

Since the discovery of PFAAs in human blood and wildlife around the year 2000, multiple human biomonitoring studies have been conducted around the world to assess the body burden of these emerging contaminants. The most extensive of all these studies are the National Health And Nutrition Examination Surveys (NHANES) conducted every two years on a representative sample of the American population. Recent data published in the "*Fourth National Report on Human Exposure to Environmental Chemicals, February 2013"* was used to generate Figures 1.2 to 1.5 for the geometric means of the four main PFAAs commonly detected in human serum samples. Figure 1.2 displays data from the five survey cycles, 1999 – 2000 *(n=1562),* 2003 – 2004 *(n=2094),* 2005 – 2006 *(n=2120),* 2007 – 2008 *(n=2100)* and 2009 – 2010 *(n=2233)* and shows a clear downward trend for serum PFOS, a slight decline for PFOA, a slight but inconsistent decrease of PFHxS, but a slightly upward trend for PFNA. In general, serum PFAA concentrations among females are lower than males, and this observation is consistent across all survey cycles (Figure 1.3). This gender specific association with serum PFAA levels may be due to regular blood loss through menstruation or sex-dependent renal elimination of these compounds or a combination of these two factors *(Zhang et al, 2013)*. When the data are stratified by race (Figure 1. 4), it is obvious that Mexican Americans (MA) have the lowest serum PFAA levels and that Non-Hispanic Whites (NHW) generally tend to have the highest levels (except for PFOS) and that the Non-Hispanic Blacks (NHB) have intermediate serum PFAA levels. No explanation is currently available in the

literature on the observed racial differences in serum levels of PFAAs. At this stage, it can only be speculated that this racial difference is a reflection of socioeconomic status (SES), which is known to be associated with serum PFAA levels (*Nelson et al. 2012).* Given the bioaccumulative nature of these toxicants, and the fact that their peak use in North America was prior to 2000, it is not surprising that older individuals (≥ 20 years) have higher serum PFAA levels compared younger ones (12- 19 years) as shown in Figure 1.5. However, this trend does not hold true for PFHxS, whereby younger people tend to have higher serum PFHxS than older Americans. PFHxS has a higher elimination half-life (8.5 years) compared to PFOS (5.4 years) *(Olsen et al. 2007),* and very little is known about is historical production volumes and trends.

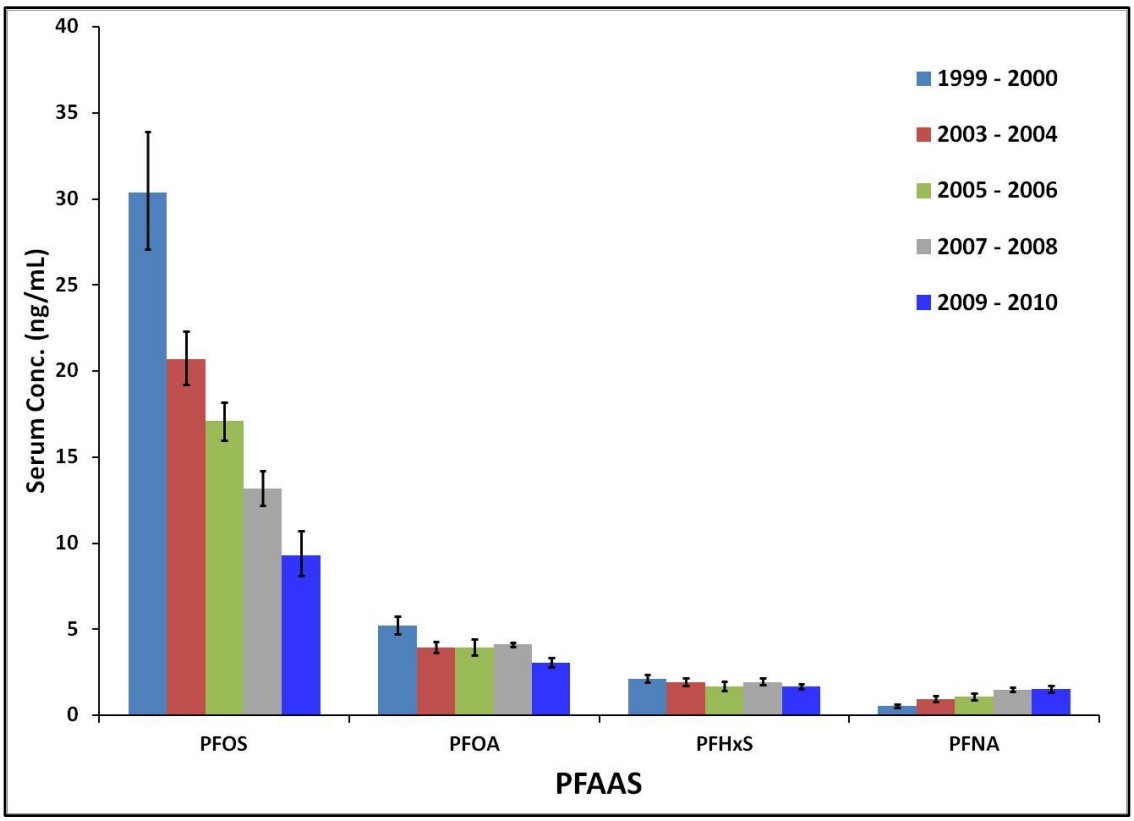


Figure 1.2 Serum levels of PFOS, PFOA, PFHxS and PFNA in the general American population based on Nutrition Health And Nutrition Examination Survey (NHANES) data. Error bars indicate 95% confidence interval. Note the consistent statistically significant decline in the serum levels of PFOS among Americans.

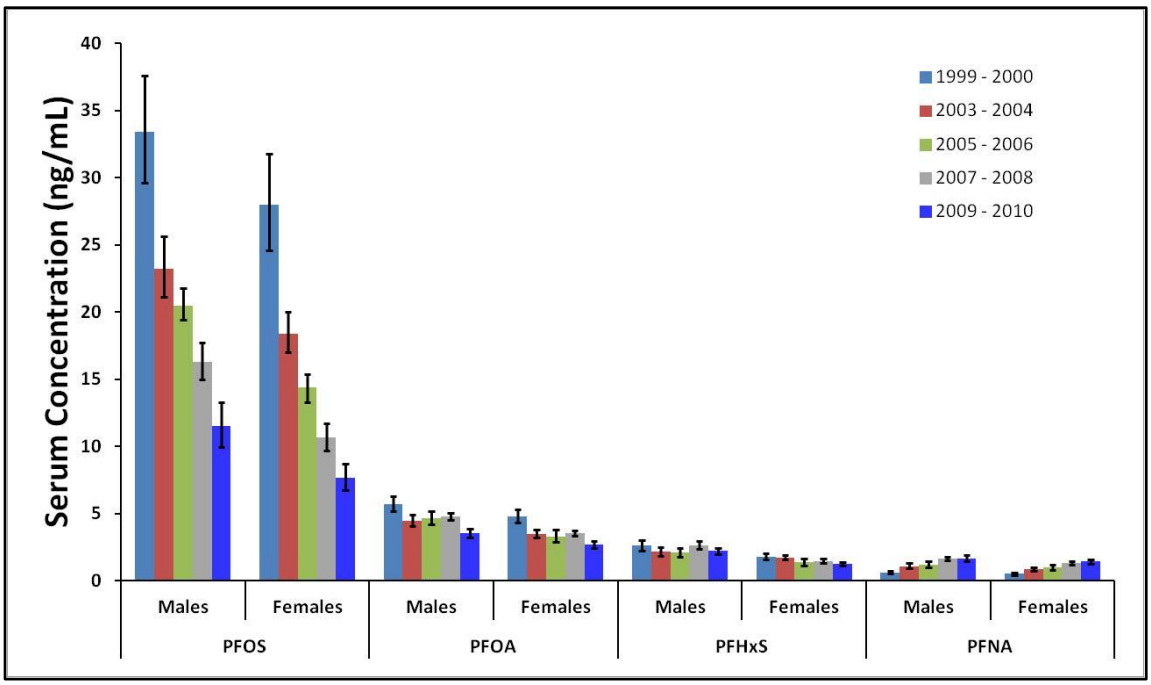


Figure 1.3 Serum levels of PFOS, PFOA, PFHxS and PFNA among Americans (based on NHANES data) stratified by gender. Notes the consistently higher levels of all four PFAAs in males compared to females. Error bars indicate 95 % confidence interval.

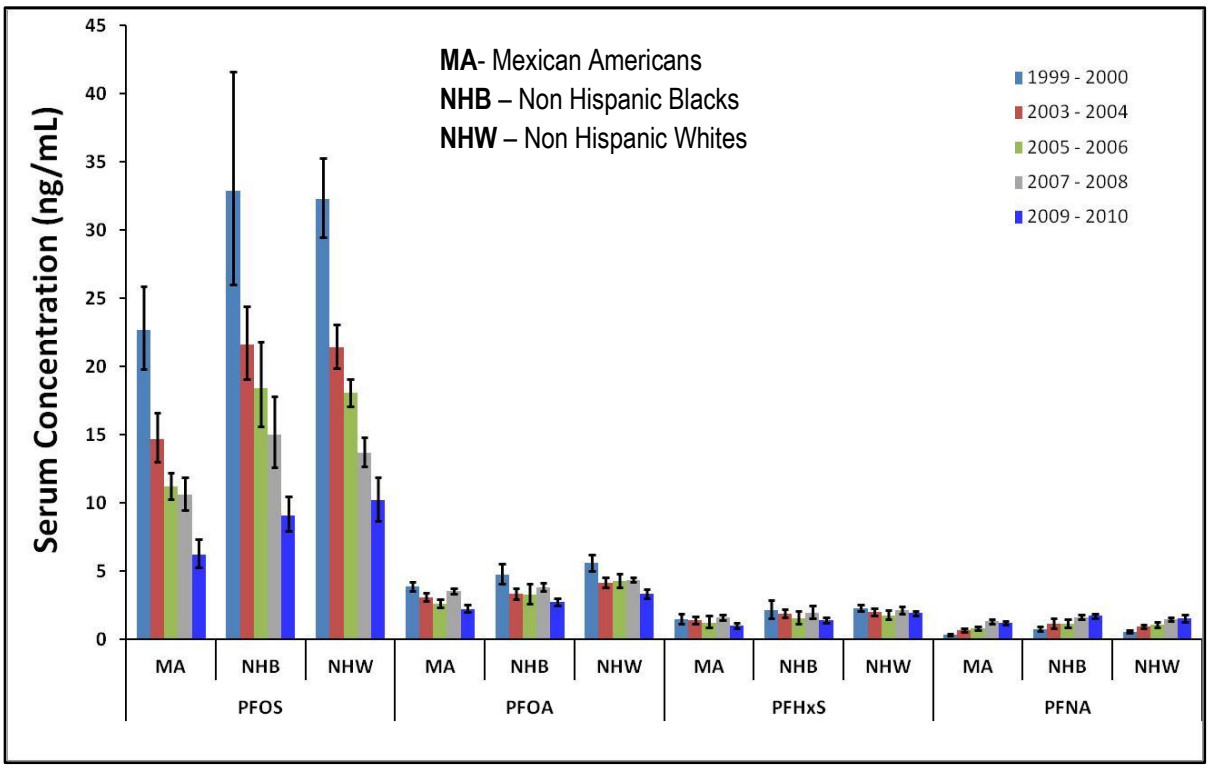


Figure 1.4 Serum levels of PFOS, PFOA, PFHxS and PFNA among Americans surveyed in the 5 NHANES study cycles. Data are stratified by race and show consistently lower serum levels of the four PFAAs among Mexican Americans. Error bars indicate 95 % confidence interval.

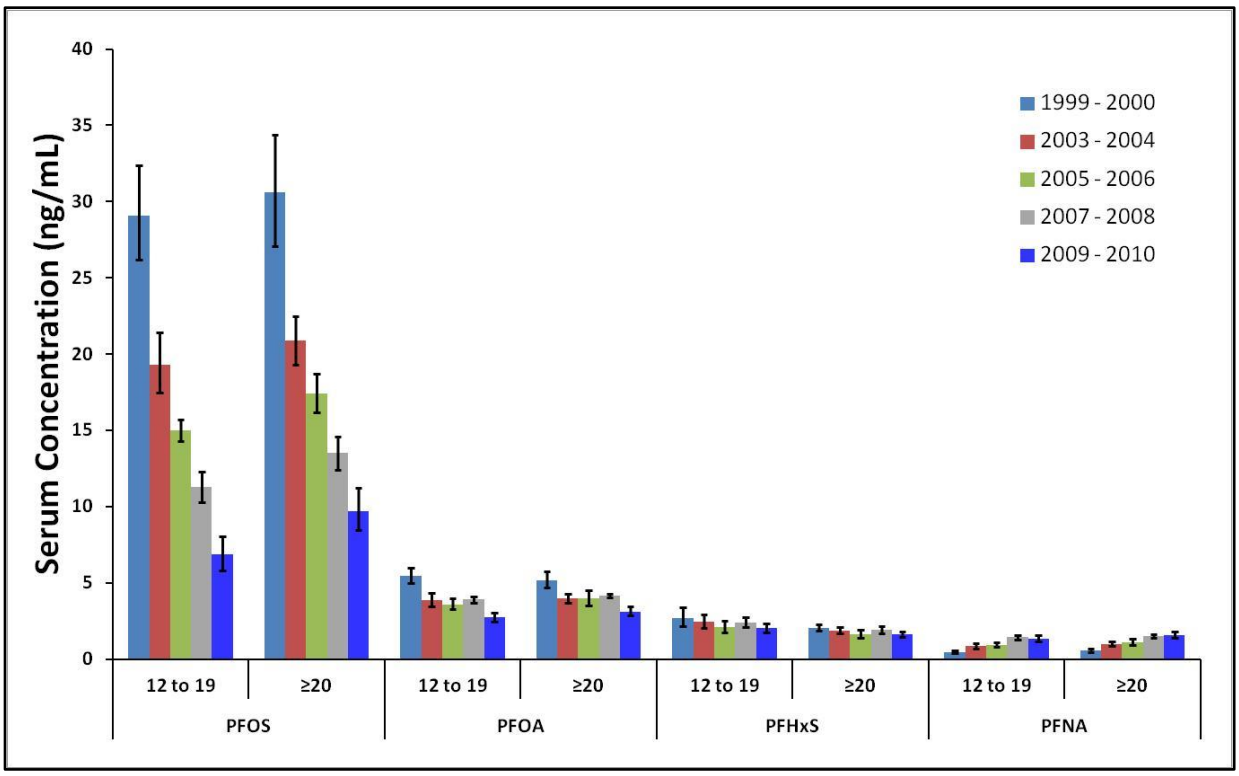


Figure 1.5 Serum levels of PFOS, PFOA, PFHxS and PFNA among Americans stratified by age. The data are from 5 consecutive NHANES survey cycles. The higher serum levels of PFOS, PFOA and PFNA in the ≥ 20 years age category compared to the 12 to 19 years category is unambiguous but not for PFHxS. Error bars indicate 95 % confidence interval.

The observed pattern of PFAA distribution in the American population, whereby the dominant PFAAs in human blood were PFOS > PFOA > PFHxS ≥ PFNA, and the gender-specific (higher in males) and age-specific (higher in the elderly) associations of serum concentrations of PFAAs have also been reported in other countries such as Canada *(Health Canada 2010; Toms et al. 2009),* Germany *(Fromme et al. 2007),* Australia *(Toms et al. 2009)* and China *(Yeung et al. 2006).*

1.8 Relative Importance of Different Pathways of Exposure

To date, at least 4 studies have attempted to assess the relative importance of the different sources and pathways of exposure and there is a general agreement that food is the major exposure source in the background population. First, *Trudel et al. (2008*) used a scenario based approach to assess exposure of the general population to PFOS and PFOA and came to the conclusion that ingestion of contaminated food and water represents the main pathway by which humans are exposed to these 2 main PFAAs. Secondly, in a thorough review of published data on PFAS intake through inhalation of both indoor and outdoor air, ingestion of household dust and dietary intake (including drinking water), *Fromme et al. (2009)* inferred that the bulk of human PFAA body burdens come from ingestion of contaminated food. Third, *Haug et al. (2011)* studied PFAA intake in a cohort of 41 Norwegian women over 4 months and came up with the median percentage intake through food for PFOA (67-84 %) and PFOS (88-99 %) which clearly demonstrates the importance of food as the major source of PFAAs exposure to humans. Finally, *Zhang et al. (2010)* compared the total daily intakes (TDI) of PFOS and PFOA from different sources in the Chinese population and they found that food accounted for 98 % of TDI of PFOA and >99% of TDI of PFOS.

Although food ingestion is the main pathway for PFAA intake in the general adult population, there is some evidence suggesting that ingestion and inhalation of contaminated dust is the most important mode of PFAA intake in children, especially in high exposure scenarios. According to the *USEPA (2008)* children can ingest up to 200 mg of dust on a daily basis compared to a much lower value

of 50 mg for adults. Thus, it was not surprising that ingestion of house dust was the main pathway of PFOS and PFOA intake by toddlers in Canada *(Shoeib et al. 2011)* and *Egeghy and Lorber (2011).* reported household dust as the main source of PFOS for American children.

1.9 Absorption and Disposition of PFAAs in Humans

Studies on dermal absorption of PFAAs in humans are practically non-existent in the literature, although one study reported that dermal absorption of PFOA is negligible *(Franko et al. 2012).* After absorption from the alimentary and respiratory tracts, the PFAAs are transported in the circulatory system to the liver, which is the major detoxification organ in the human body. While a considerable fraction of the PFAAs are retained in the liver, PFAAs also circulate in blood serum, bound to proteins including serum albumin *(Han et al. 2003; Jones et al. 2003; Luo et al. 2012)*. Unlike other Persistent Organic Pollutants (POPs) which are known to be lipophilic, the PFAAs can actually be described as lipophobic, and seem to be proteinophillic. There is currently no evidence that PFAAs can be metabolized in the liver, or in any organ, and the primary disposition of PFAAs in the liver is hepatocyte excretion to bile *(Harada et al. 2007)*. Free PFAAs can then be reabsorbed from the small or large intestine back into portal blood flow (back to the liver) and the cycle repeats itself, a process known as enterohepatic circulation *(Lau et al. 2007)*. Thus the extent of PFAA elimination through fecal excretion is minimized, supported by rodent data *(Benskin et al. 2009a)* and human data *(Beesoon et al. 2012).*

Studies directed at understanding the different pathways by which humans excrete PFAAs are rare, likely because the concentrations in urine and feces are very low, owing to long elimination half-lives (2.5 to 8.5 years) *(Olsen et al. 2007).* However, recent papers indicate that renal clearance may be the most important elimination route in the general population *(Beesoon et al. 2012; Zhang et al. 2013).* Pregnant women may also eliminate a significant proportion of their PFAA body burdens through the placental barrier into fetal circulation, while breast-feeding mothers transfer additional PFAAs to their babies through breast milk *(Kim et al. 2011; Kubwabo et al. 2013; Liu et al. 2011; Pratt et al. 2013).*

1.10 PFAAs and Human Health

Research looking at potential adverse health effects of PFAAs can be divided into 2 broad categories. First, there are the fundamental laboratory based toxicology studies on either cell lines or with live animals such as rats, mice, monkeys and fish. Second, epidemiological population-based studies are conducted which can demonstrate exposure-disease associations through various study designs such as ecological, cross-sectional, case-control and prospective follow-up.

1.10.1 Laboratory Based Toxicology Studies

Laboratory toxicology studies reveal six major pathologies associated with PFAAs: carcinogenicity, hepatotoxicity, neurotoxicity, developmental toxicity, immunotoxicity and disruption of the endocrine system. Although these studies are summarized below under distinct headings, they are not mutually exclusive and substantial overlap exists among them. For example, studies classified under

"disruption of endocrine system" can be equally classified under "developmental toxicity" for all intents and purposes.

1.10.1.1 Carcinogenicity

Although there is a fair amount of data on the carcinogenic potential of PFOS and PFOA in the literature, such data for the other PFAAs are scanty. According to the *OECD (2002)* report that described an older study conducted by the 3M Company, PFOS can cause a significant increase in hepatocellular adenomas, follicular cell adenomas, and tumors of the mammary gland in Sprague-Dawley rats fed 20 µg/g body weight/day over a period of 104 weeks. An increased incidence of Leydig cell adenoma in male rats fed ammonium perfluorooctanoate (APFO) was reported in three independent studies by *Butenhoff et al. (2012), Biegel et al. (1995)* and *Kennedy et al. (2004),* showing some consistency across studies.

1.10.1.2 Hepatotoxicity

Given the primary role that the liver plays in detoxification, and its rich supply of blood, it is commonly the target of xenobiotic insults, and PFAAs are no exception. Enlargement of the liver and/or increase of liver weights are common adverse effects of PFAAs in laboratory animals. Data on hepatotoxicity is available not only for PFOS *(OECD 2002)* and PFOA *(Kennedy et al. 2004)* but also for perfluorobutanesulfonate (PFBS) (*Ehresman et al. 2007),* perfluorohexanesulfonate (PFHxS) *(Ehresman et al. 2007*), perfluorobutyrate (PFBA)*(Foreman et al. 2009*), perfluorohexanoate (PFHxA)(*Kudo et al. 2006*),

perfluoroheptanoate (PFHpA) (*Kudo et al. 2006*), perfluorononanoate (PFNA)*(Kudo et al. 2006; Van Rafelghem et al. 1987)* and perfluorodecanoate (PFDA)*(Van Rafelghem et al. 1987).*

1.10.1.3 Neurotoxicity

The first report drawing attention to the potential neurotoxic effects of PFAAs was probably the one conducted by *Austin et al. (2003)* on adult female rats injected intraperitoneally with PFOS. In this study, a dose dependent increase of norepinephrine was found in the hypothalamus of treated rats. *Onishchenko et al. (2011*) chronically exposed pregnant mice to 0.3 mg/kg body weight of PFOS or PFOA, and reported that PFOS levels in the brains of the newborn mice were 4 times higher than PFOA, suggesting that PFOS may be more efficient than PFOA at crossing the blood-brain barrier. In the same context, *Maestri et al. (2006*) analyzed PFOS and PFOA in pooled human tissue samples and reported PFOS/PFOA concentration ratios of 1.7 and 2.5 in blood and brain respectively, suggesting that PFOS may be more efficient at crossing the blood-brain-barrier compared to PFOA. This is validated by the work of *Harada et al. (2007)* who analyzed paired serum and cerebrospinal fluid from 7 individuals and found that the PFOS concentrations were consistently higher than PFOA in all of the participants, PFOA being undetectable (LOD 0.04 ng/mL) in 5 out of the 7 patients. More recently, *Zeng et al. (2011)* exposed pregnant Sprague Dawley rats to 0.1, 0.6 and 2.0 mg/kg b.w. of PFOS and showed that the ultrastructure of synapses in the offspring's brain were adversely affected, with a reduced number of synaptic vesicles, shorter active synaptic zones and decreased synaptic

curvature. Moreover, they also reported that mRNA levels of proteins associated with the synaptic vesicles, namely synapsin 1, synapsin 2 and synaptophysin, were significantly reduced.

1.10.1.4 Immunotoxicity

Multiple laboratory studies have been conducted over the past decade to verify if PFAAs (in particular PFOS and PFOA) can cause immunotoxicity. The first report about the potential immunotoxicity of PFOA came from the group of *Yang et al. (2001)* who showed that mice exposed to PFOA developed major thymic and splenic atrophies accompanied with drastic reduction of the number of viable thymocytes and splenocytes. *Dewitt et al. (2008;2009)* were also major contributors to knowledge on PFOA induced immunotoxicity in mice. Specifically, compared to controls, IgM antibody production was suppressed in a dose-dependent manner by PFOA, but IgG titers went up for mice treated at 3.75 and 7.5 mg/kg/day. *Peden-Adams et al. (2008)* were the first to describe the immunotoxic potential of PFOS, which was attributed to a deregulation of the immunocompetency of B-cells in mice exposed to PFOS.

1.10.1.5 Endocrine Disruption

One of the endocrine systems that PFAAs are believed to affect, and which has been extensively studied in lab animals, is thyroid function. In 2002, *Seacat et al. (2002)* published the first study documenting a possible adverse effect of PFOS on circulating thyroid hormone levels in Cynomolgus monkeys. In that study, nearly 50 % reduction of circulating triiodothyronine (T3) was observed in both

males and females exposed to 0.75 mg/kg/day, but a less consistent reduction of thyroxine (T4) (only in females) was noted. However, the reduction in the thyroid hormone levels was not accompanied by a proportional compensatory increase in the thyroid stimulating hormone (TSH) levels, which did not suggest the classical feature of hypothyroidism (decrease in T3 and T4 with increase in TSH levels as a result of activation of the hypothalamic-pituitary-thyroid (HPT) negative feedback mechanism). Thus, "hypothyroxinemia" rather than "hypothyroidism" would be a most appropriate term to explain this hormonal imbalance. The underlying biochemical mechanism of PFOS-induced hypothyroxinemia has recently been scrutinized by multiple studies and hypotheses involving de novo production of thyroid hormones, or disruptions of the HPT axis, have both been dismissed by one group of authors in light of new evidence that bioassays for thyroid hormones are biased by presence of the PFAAs *(Chang et al. 2007; 2008; 2009).*

Unlike for PFOS, toxicological data is still sparse on the possible adverse effects of PFOA on thyroid status of laboratory animals. To date, there are two commonly cited studies in the literature addressing this knowledge gap for PFOA. In a chronic toxicity study of ammonium perfluorooctanoate in Cynomolgus monkeys, *Butenhoff et al. (2002)* found a statistically significant reduction in T3 levels (but not for T4 nor TSH) after 5 week treatment with 30 mg/kg/day of PFOA, at which time the serum level of PFOA reached 158 µg/mL.

1.10.1.6 Developmental Toxicity

The most overt developmental endpoint observed when rodents are exposed to high levels of PFOS or PFOA is mortality of the fetus or newborn. *Lau et al.*

(2003) exposed female rats to 0, 1, 2, 3, 5 and 10 mg/kg of PFOS and the pups were followed prospectively after birth. A survival rate of 50% was noted for the 3 mg/kg treatment group, showing a dose-dependent relationship with PFOS body burden. The same phenomenon was reported earlier by *Grasty et al. (2003).* Over and beyond excess neonatal mortality associated with maternal exposure to high doses of PFOS, multiple other adverse development endpoints are reported in the literature. Examples are low birth weight, cleft palate, developmental abnormalities of the heart, fragile bones *(Lau et al. 2003),* thickening of alveolar walls in the lungs *(Grasty et al. 2005)* and developmental neurotoxicity *(Slotkin et al. 2008).*

1.10.2 Epidemiological Studies.

1.10.2. 1 Mortality

Mortality attributable to PFOA and PFOS has been investigated in occupational cohorts. *Leonard et al. (2008)* conducted a retrospective follow-up of 6,027 men and women who worked at the DuPont Washington Works (WW) polymer production facility (using PFOA as the main raw material) in Parkersburg, West Virginia. When mortality in the exposed cohort is compared to the general US population and the State of West Virginia, the data suggested that there is lower than expected mortality, reaching statistical significance ($p<0.05$) for all causes of death, all malignant neoplasms, cancer of the digestive system, respiratory system, lung, stomach and prostate. Curiously, when the exposed cohort was compared to an unexposed cohort working for the same company, the Standardized Mortality Ratios (SMRs) data suggest an excess mortality in the

exposed cohort for a whole range of cancers but statistical significance was never reached. Using the same worker population as above, *Sakr et al. (2009)* studied 4747 workers and looked at ischemic heart disease (IHD) associated mortality, using proportional hazards regression models and came up with the conclusion that exposure to PFOA was not a risk factor for IHD mortality. More recently, in 2012, a third study (*Steenland and Woskie 2012)* in the same manufacturing facility followed 1308 workers from 1979 to 2004, and found statistically significant increases in the cause-specific mortality for mesothelioma [SMR *(95% CI)*: 2.85 (1.05 *to* 6.20)], diabetes mellitus [SMR *(95% CI)*: 1.90 (1.35 *to* 2.61)] and chronic renal disease [SMR *(95% CI)*: 2.85 (1.66 *to* 5.32)*]*. The only study to date looking at mortality in an occupational cohort exposed to PFOS was conducted on 2083 employees working for the 3M production facility in Decatur, Alabama *(Alexander et al. 2003)*. Of the 145 overall deaths in this cohort, only 65 were from workers with a "high" exposure profile. There were 2 deaths from liver cancer among workers with at least one year of exposure to PFOS. Three bladder cancer deaths among those with a high exposure profile occurred during the follow *to* up period giving a high Standard Mortality Rate of 12.77 (*95% CI* 2.63 *to* 37.35).

1.10.2. 2 Exposure to Disease Association Studies

As a direct extension of toxicological research, public health professionals have taken the research agenda a step further to examine for any impacts in background populations, as well as in highly exposed communities. In an effort to maintain consistency of the discussion, the exposure-disease association studies will be

grouped similarly, but not exactly, to the review of toxicological studies. The following groups of clinical outcomes will be discussed: endocrine dysfunction, higher probability of cancer, hypercholesterolemia and cardiovascular disease, abnormal liver function, hyperuricemia, diabetes, neurobehavioral disorders, lower reproductive abilities and poor developmental outcomes.

1.10.2.2.1 Endocrine Dysfunction

Using medical surveillance examination data of 3M employees from Decatur, Alabama (n=263), and Antwerp, Belgium (n=255), *Olsen et al. (2003)* reported no significant associations between thyroid hormones (TSH, T4, free T4 and T3) and PFOS or PFOA exposure, except that the mean T3 level in the highest quartile of PFOS/PFOA category was statistically significantly higher than that of the lowest quartile of the PFOS/PFOA category. In a study on 371 community residents of Little Hocking, West Virginia, whose drinking water supply was heavily contaminated with PFOA *(Nolan et al. 2009)* at a median concentration of 5.7 µg/L (range: 1.7 – 17.1 µg/L) (compared to a median of 0.22 µg/L (range: 0.17 *to* 0.24) in another water facility in the same county), *Emmett et al. (2006)* did not find any association between serum PFOA levels and TSH (the only thyroid hormone monitored). *Olsen and Zobel (2007)* made a cross-sectional analysis of 506 workers in 3 different 3M PFOA manufacturing facilities (Decatur (Alabama), Cottage Grove (Minnesota) and Antwerp (Belgium) and reported no associations between TSH or total T4 with serum PFOA but a negative association between free T4 and serum PFOA, and a positive association for T3. *Melzer et al. (2010)* used NHANES data from 1999 to 2006, which includes

PFAA data on 3974 adults and associated information on the presence or absence of thyroid disease (n=163, 117 F and 46 M). Multiple logistic regression models showed that women in the highest PFOA quartile and men in the highest PFOS quartile were more likely to have a diagnosis of thyroid disease compared to the lowest two quartiles, with adjusted odds ratios of 2.24 (*95% CI*: 1.38 *to* 3.65) and 2.68 (*95% CI*: 1.03 *to* 6.98), respectively. *Chan et al. (2011)* conducted a case - control study on 96 hypothyroxinemic pregnant women (defined as having normal TSH levels and the lowest 10^{th} percentile of free T4 from a cohort of 974 individuals) and 175 matched controls (normal TSH and free T4 between 50^{th} and 90^{th} percentile) in Alberta, Canada. The authors concluded that there was no association between PFOS, PFOA, and PFHxS exposure and hypothyroxinemia. A study conducted on 551 Taiwanese participants (214 males and 337 females) by *Lin et al. (2013),* whereby four different PFAAs (PFOS, PFOA, PFNA and PFUA) and free T4 and TSH were measured in the serum samples, showed a statistically significant positive association between serum PFNA and free T4 (*p-trend*=0.012). Surprisingly, elevated serum free T4 was not accompanied by a decrease of TSH in this cohort. The absence of consistency across the different studies, the low magnitudes of the association and the cross-sectional nature of these studies (with the exception of the *Chan et al. (2011)* study where no statistical result was noted), it would be risky to suggest a causal link between PFAA exposure and thyroid hormone disruption.

Lopez-Espinosa et al. (2011) did a cross-sectional study on 3076 boys and 2931 girls aged 8 to 18 years from the Mid-Ohio Valley community, with primary

predictors of interest being serum PFOS and PFOA, and with the outcome variable being age at onset of puberty. Higher serum PFOS in boys was associated with reduced odds of reaching puberty with a delay of 190 days between highest and lowest quartiles of serum PFOS. Delayed menarche in girls was associated with high serum levels of both PFOS (138 days between highest and lowest quartile) and PFOA (130 days between highest and lowest quartile). A recent study by *Joensen et al. (2013)*, reported a statistically significant negative association between serum PFOS and total testosterone and the following calculated parameters: free testosterone (FT), free androgen index (FAI), ratios of FAI/LH, T/LH and FT/LH (LH: Luteinizing Hormone). No association was reported between any PFAA and semen quality in this study.

1.10.2.2.2 Cancer

Associations (or their absence) between PFOS and/or PFOA exposure and cancer of the bladder, prostate, digestive system, respiratory systems in occupational cohorts have been discussed in the section "mortality". Using a prospective follow-up study design looking at plasma levels of PFOS and PFOA, *Eriksen et al. (2009)* recruited 713 cases of prostate cancer, 332 cases of bladder cancer, 128 cases of pancreatic cancer and 67 cases of liver cancer and 772 controls in the general Danish population. Based on the crude and adjusted incidence rate ratios (IRRs) calculated for each type of cancer, the authors concluded that none of the four above mentioned cancers are associated with plasma levels of PFOS or PFOA, albeit a non-statistically significant increase in the IRR for prostate cancer was found in the three upper quartiles compared to the lowest quartile. A recent

case-control study by *Bonefeld-Jorgensen et al. (2011)* in the Greenland Inuit population from 2000 to 2003 recruited 31 breast cancer cases and 115 controls. A broad spectrum of persistent organic pollutants as well as certain metals were measured in the plasma of participants and a statistically significant but very modest association (OR = 1.03 (*95% CI*: 1.00 *to* 1.07)) was found between plasma PFOS levels and a diagnosis of breast cancer.

1.10.2.2.3 Neurobehavioral disorders

To date there are at least 3 studies in human populations looking at exposure to PFAAs and neurobehavioral disorders as the outcomes of interest. First, *Hoffman et al. (2010)* studied 571 children aged 12 to 15 years among the 1999 *to* 2000 and 2003-2004 NHANES participants of whom 48 had a previous medical diagnosis of attention deficit hyperactivity disorder (ADHD) as reported by the parent. Serum concentrations of PFOS, PFOA, PFHxS and PFNA were the dependent (predictor) variables and statistically significant positive associations were found for all PFAAs except for PFNA and ADHD. The second study, by *Stein and Savitz (2011)* was conducted in the Mid-Ohio community-study 10,546 children 5 to 18 years of age of whom 1308 reported ADHD and 538 reported ADHD with medication use. Similar to *Hoffman et al. (2010),* the highest statistically significant adjusted odd ratio (comparing the highest quartile to the lowest quartile) detected was for PFHxS (OR=1.69 CI: 1.21 *to* 2.08). The third study by *Gump et al. (2011)* attempted to quantify "impulsivity" by measuring inter-response times (IRTs) in a group of 83 children (30 girls, 53 boys) and associations with serum PFAAs. The authors reported a statistically significant

decrease in IRTs with increasing serum levels of PFHxS and PFOS, suggesting higher impulsivity (characteristic of ADHD) was associated with serum PFHxS and PFOS.

1.10.2.2.4 Cholesterol and Cardiovascular Disease Risk

Although a few animal toxicology studies have documented hypolipidemic effects of PFOS and/or PFOA, most of the studies on human populations do not reflect this effect. Analysis of the 2003-2004 NHANES data by *Nelson et al. (2010)* found statistically significant positive associations between total cholesterol and non-high density lipoprotein (non-HDL) cholesterol and PFOS and PFNA. For PFOA there was also a positive association, though it was not statistically significant at the 95 % confidence interval level. Interestingly, PFHxS appeared to have a protective effect on the lipid profiles, with statistically significant negative associations between: (A) serum PFHxS and total cholesterol (β-coefficient : –0.93 mg/dL (95 % C.I:–1.80 *to* –0.06), (B) serum PFHxS and non-HDL cholesterol (β-coefficient: –1.13 mg/dL (95 % C.I: –1.90 *to* –0.35) and (C) serum PFHxS and LDL-cholesterol (β-coefficient: –2.06 mg/dL (95 % C.I: –3.54 to –0.58). *Frisbee et al. (2010)* conducted a cross-sectional study on children and adolescents (n=12476) in the Mid-Ohio River Valley community having a high exposure to drinking water contaminated with PFOA *(Nolan et al. 2009).* Multiple linear regression models show that total cholesterol and low density lipoprotein (LDL) cholesterol showed strong (p<0.001 in all models) positive linear associations with both PFOS and PFOA. Serum triglycerides also showed a positive association with PFOA, with a p-value of 0.02, but not with PFOS.

Steenland et al. (2009) surveyed 46,294 adult community residents affected by contaminated drinking water who were not on any cholesterol-lowering drugs. With the lowest quartiles of PFOS as referents, the odds *(95% CI)* of higher total cholesterol for the second, third and fourth quartiles were 1.13 (1.04 *to* 1.22), 1.28 (1.19 *to* 1.39) and 1.51 (1.40 *to* 1.64) respectively. Similarly for PFOA, the odds *(95% CI)* of higher total cholesterol for the second, third and fourth quartiles were 1.21 (1.12 *to* 1.31), 1.33 (1.23 *to* 1.43) and 1.40 (1.29 *to* 1.51) respectively. Another cross-sectional study by *Eriksen et al. (2013)* on a Danish cohort of 663 men and 90 women, aged between 50 and 65, again found significant positive associations between total cholesterol and PFOA (increase of 4.4 (*95% CI*: 1.1 *to* 7.8) mg/dL of total cholesterol with each quartile increase in serum PFOA) and PFOS (increase of 4.6 (*95% CI*: 0.8 *to* 8.5) mg/dL of total cholesterol with each quartile increase in serum PFOS) in models adjusted for education, age and lifestyle. Based on data (n=2345) generated by the Canadian Health Measures Survey (CHMS), the only large scale national biomonitoring study conducted in Canada to date, *Fisher et al. (2013)* reported statistically significant positive associations between total cholesterol and serum PFHxS, but not for PFOS nor PFOA. *Shankar et al. (2012)* examined medical data of 1216 participants (593 males and 623 females) of the 1999 to 2003 NHANES surveys and tested the hypothesis that serum PFOA is a risk factor of cardiovascular disease in the study population. With quartile 1 of PFOA exposure as the reference, the odds *(95% CI)* of having cardiovascular disease and peripheral arterial disease for participants in

quartile 4 was 2.01 (1.12 *to* 3.60; *p-trend*=0.01) and 1.78 (1.03 *to* 3.08; *p-trend*=0.04), respectively.

1.10.2.2.5 Diabetes and Metabolic Syndrome

Although animal toxicology studies linking PFAA exposure to diabetes are non-existent in the literature, to date there are two major epidemiological papers on the association between PFAAs and diabetes. First, *Lin et al. (2009*) analyzed NHANES 1999 2000 and 2003 2004 data from 474 adolescents and 969 adults using multiple linear regression models to estimate the effect of different PFAAs on blood glucose, insulin and homeostasis model assessment of insulin resistance (HOMA-IR) and β-cell function. Overall, there was a statistically significant positive association between PFNA and blood glucose levels [OR=3.16 *95 % CI*: 1.39 *to* 7.16], yet negative associations for PFNA with HDL-cholesterol levels [OR=0.67 *95 % CI*: 0.45 *to* 0.99] and presence of metabolic syndrome [OR=0.37 *95 % CI*: 0.21 *to* 0.64]. Serum PFOA was positively associated with β-cell function (β-coefficient = 0.07 ± 0.03, p <0.05). PFOS levels were positively associated with blood insulin (β-coefficient = 0.14 ± 0.05, p<0.01), HOMA-IR (β-coefficient = 0.14 ± 0.05, p<0.001), β-cell function (β- coefficient = 0.15 ± 0.05, p<0.01) and serum HDL-cholesterol [OR=1.61 *95% CI:* 1.15 *to* 2.26]. The second study by *MacNeil et al. (2009)* was case-control by design with 1055 cases of diabetics and 12,867 controls from the Mid-Ohio River Valley residents. The primary predictor of interest was serum PFOA and type II diabetes was the outcome variable. Regression analysis showed no association between PFOA levels and either type II diabetes or fasting plasma glucose levels.

1.10.2.2.6 Immune System Disorders

Although laboratory toxicology studies suggest possible immunotoxic effects of PFAAs, there seems to be a general lack of agreement among human epidemiology studies on such effects. *Okada et al. (2012)* conducted a prospective cohort study on pregnant Japanese women (n=343) to verify if prenatal exposure to PFOS and/or PFOA can affect the immune status of babies or infants. The only statistically significant finding in this study was that high maternal PFOA levels were associated with a decrease in cord blood immunoglobulin E (IgE) levels. *Wang et al. (2011)* conducted a case-control study looking at atopic dermatitis (43 cases, 201 controls) as the outcome of interest, at age 2 as part of the Taiwan Birth Panel Cohort, and cord blood levels of PFOS and PFOA as the predictor variables. Unlike the findings of *Okada et al. (2012)*, this study found a positive correlation between serum IgE levels and PFOS and PFOA using multiple linear regressions on the log transformed data. Multiple logistic regression on the atopic dermatitis (i.e. Yes/No) and cord serum PFAAs data do not indicate any statistically significant association.

Fei et al. (2010) used serum PFOS and PFOA data from 1400 pregnant mothers recruited in the 1996-2002 Danish National Birth Cohort and looked prospectively at the risk of hospitalization of the offsprings until 2008. The authors reported no association between prenatal exposure to PFOS/PFOA and risk of hospitalization. The most recent research by *Grandjean et al. (2012)* used a prospective cohort study design to examine 656 babies born in the period 1997-2000 in the Faroe Islands, followed up through 2008. Maternal serum PFOS

concentration (range: 23.2 *to* 33.1 ng/mL) were negatively associated with the child's diphtheria antibody concentration: a 2-fold increase in maternal serum PFOS was associated with a 39 % (*95% CI*: 17% to 55%) decrease in diphtheria antibody levels at age 5. More importantly, a doubling of the serum PFOS (range: 13.5 to 21.1 ng/mL) and PFOA (range: 3.33 *to* 4.96 ng/mL) concentrations of the child at age 5 increased the odds (range: 2.38 *to* 4.20) of having their tetanus and diphtheria antibodies falling below the clinically protective level of 0.1 IU/mL at age seven.

1.10.2.2.7 Hyperuricaemia

Hyperuricemia is a pathological condition whereby the concentrations of uric acid in the blood exceed the normal levels (i.e. 2.4 *to* 6.0 mg/dL for females and 3.4 *to* 7.0 mg/dL for males). To date, four major studies have reported on possible associations between serum PFAA levels and uric acid. *Sakr et al. (2007)* studied 1025 workers in an ammonium perfluorooctanoate (APFO) manufacturing facility and used multiple linear regressions to find a potential association between serum PFOA and uric acid. In a study on medical surveillance of workers routinely working with PFOA (exposed) and executive clerks and other blue-collar workers (controls), *Costa et al. (2009)* reported a statistically significant difference (p=0.039) between the mean uric acid levels between 34 exposed (6.29 mg/dL uric acid) and 34 matched controls (5.73 mg/dL uric acid). In a cross-sectional study of 54,951 residents in Ohio and West Virginia exposed to high levels of PFOA in their drinking water, *Steenland et al. (2010)* showed a definite (p<0.0001) but moderate trend in the serum uric acid levels of the research

participants. *Geiger et al. (2013)* used data from NHANES surveys of 1999-2000 and 2003-2008 and performed a cross-sectional analysis of 1772 participants 18 years and younger, and found statistically significant positive associations between serum PFOS or PFOA levels and hyperuricemia.

1.10.2.2.8 Abnormal Liver Clinical Chemistry Parameters

The routine battery of liver function tests (LFTs) includes total bilirubin (TB), alkaline phosphatase (Al. P), alanine transaminase (ALT), aspartate transaminase (AST) and gamma glutamyl transferase (γ-GT). Using medical surveillance data on occupational cohorts in three different chemical manufacturing plants, *Olsen et al. (2003)* reported that 23% of the male employees in the highest PFOS quartile had at least one LFT above the reference range compared to 14%-16 % in the three lower quartiles. In a more recent prospective longitudinal study, *Olsen et al. (2012)* followed an occupational cohort of 170 men and 9 women working in physical demolition of 3M PFAA manufacturing facilities. Using four different linear regression models to assess how changes in the serum PFOS/PFOA concentrations over time can affect the changes in hepatic clinical chemistry parameters, the authors did not find any statistically significant associations. A more recent large scale biomonitoring study by *Gallo et al. (2012)* on the highly exposed community (*n*=47,092) in the Mid-Ohio River Valley reported statistically significant positive associations in linear regression models between ln-ALT and ln-PFOA (β-coefficient=0.022, *95% CI*: 0.018 *to* 0.025) and ln *to* PFOS (β-coefficient=0.020, *95% CI*: 0.014 *to* 0.026). *Lin et al. (2010)* analyzed data (n=2216) from the publicly available NHANES database for the 1999-2000

and 2003-2004 cycles and reported a statistically significant increases of serum ALT by 1.86 IU/L (*95% CI*: 1.24 *to* 2.48) and serum γ-*G*T by 0.08 IU/L (*95% CI*: 0.05 *to* 0.11). The positive associations between serum PFOS/PFOA and liver enzymes were accentuated in obese participants and those with insulin resistance and/or having a positive diagnosis of metabolic syndrome.

1.10.2.2.9 Chronic Kidney Disease

Shankar et al. (2011) merged data from four NHANES cycles (1999-2000, 2003-2004, 2005-2006 and 2007-2008) to test the hypothesis that plasma PFOS and PFOA were associated with chronic kidney disease (CKD), with the following case definition: *a glomerular filtration rate (GFR) less than 60 mL/minute/1.73 m^2*. Plasma PFOA and PFOS levels were stratified into quartiles and ORs for CKD were calculated with the first quartile as the referent. The adjusted ORs (95 % CIs) for quartiles 2, 3 and 4 of PFOA exposure (*p-trend*=0.015) and PFOS exposure (*p-trend*=0.019) were progressively elevated for higher quartiles. Given the cross-sectional nature of the data, whereby temporality cannot be established, and the fact that statistical significance is only reached at the fourth quartile, we believe that this putative PFOA/PFOS - CKD association reported in this study is questionable. *Watkins et al. (2013)* used the Mid-Ohio River Valley exposed community database to perform a cross-sectional analysis of the data and performed a longitudinal study using a predictive model for "PFOA at birth" and follow-up for CKD incidence. Although the serum PFOS, PFOA and PFHxS was cross-sectionally associated with lower estimated GFR (eGFR), as in the *Shankar et al. (2011)* study, follow-up data do not show any relation between PFOS and

eGFR. To make sense of these conflicting findings, the authors proposed that the association between serum PFOA and CKD found in cross-sectional studies may be due to reverse causation; that is; the high PFOA found in CKD patients may be a consequence of CKD, rather than the cause of it.

1.10.2.2.10 Osteoarthritis (OA) and Endometriosis

Using the database of the Mid-Ohio River Valley, *Innes et al. (2011)* identified 3,731 cases of adults with osteoarthritis and 45,432 controls. Using the first quartile of PFOA exposure as the referent, the adjusted ORs *(95 % CI)* of OA for the second, third and fourth quartiles were 1.16 (1.03 *to* 1.31), 1.21 (1.07 *to* 1.36) and 1.42 (1.26 *to* 1.59), showing a consistent increasing risk of OA with higher serum levels of PFOA (*p-trend*=0.00001). In the case of PFOS, the directionality of the association is opposite to that seen for PFOA, with adjusted ORs *(95 % CI)* of OA for the second, third and fourth quartiles of PFOS exposure being 0.91 (0.81 *to* 1.01), 0.94 (0.84 *to* 1.06) and 0.76 (0.68 *to* 0.85) with respect to the first quartile.

Endometriosis is a rather common health issue in women, whereby the endometrium grows beyond the uterus and invades other tissues such as the ovaries, Fallopian tubes, and the lining of the pelvic cavity. Infertility is a quite common complication associated with endometriosis. *Louis et al. (2012)* conducted a cross sectional study from 2007 to 2009 in the Salt Lake City or San Francisco areas, and statistically significant adjusted odd ratios *(95% CI)* between endometriosis and serum PFOA [1.89 (1.17 *to* 3.06)], and PFNA [2.20 (1.02 *to* 4.75)] were found. However, when parity was included in the multiple logistic

regression model, the statistical significance was slightly attenuated with adjusted ORs *(95% CI)* of 1.62 (0.99 *to* 2.66) and 1.99 (0.91 *to* 4.33) for PFOA and PFNA respectively. The odds *(95% CI)* of moderate/severe endometriosis were also increased with higher PFOS 1.86 (1.05 *to* 3.30) and PFOA 2.58 (1.18 *to* 5.64) serum concentrations, which were also weakened by parity adjustment.

1.10.2.2.11 Adverse Effects on the Reproductive System

Knowledge that the human reproductive system can be adversely affected by environmental toxicants such as polychlorinated biphenyls (PCBs) *(Chevrier et al. 2013; Hauser 2006; Yang et al. 2008)*, polybrominated diphenyl ethers (PBDEs) *(Abdelouahab et al. 2011; Chevrier et al. 2013)*, polychlorinated dibenzodioxins (PCDDs) *(Cok et al. 2008)* and polychlorinated dibenzofurans (PCDFs), has spurred multiple environmental epidemiology studies to look at potential adverse reproductive effects of PFAAs in human populations. *Fei et al. (2009)* were among the first to investigate the likelihood that serum PFOS and PFOA may impact fecundity. Based on a study of 1240 pregnant women from the Danish National Birth Cohort the authors reported statistically significant associations between maternal levels of PFOA and PFOS and time to pregnancy (TTP) with $p<0.001$ in both cases. Subfecundity, as the primary outcome of interest, was also investigated by *Whitworth et al. (2012)* using a case-control study design on a Norwegian cohort of 416 subfecund women (TTP>12 months) and 494 controls. For parous women, the odds *(95% CI)* of subfecundity for the highest quartile of PFOS and PFOA exposure, compared to the lowest quartile, were 2.1 (1.2 *to* 3.8) and 2.1 (1.0 *to* 4.0), respectively. *Vestergaard et al. (2012)*

investigated the possible link between serum PFAAs and TTP on 222 Danish women attempting to conceive for the first time. The adjusted odds *(95 % CI)* of subfecundity for "median and above median" exposure levels of PFOS, PFOA, PFHxS and PFNA were 0.98 (0.54 *to* 1.77), 1.21 (0.67 *to* 2.18), 0.67 (0.37 *to* 1.20) and 0.67 (0.37 *to* 1.25) respectively, which do not support the hypothesis that exposure to PFAAs is associated with subfecundity.

Sperm quality, as a surrogate of reproductive toxicity in human populations, was investigated by *Raymer et al. (2012)* in a cross-sectional study on 256 men in Durham, North Carolina, from 2002 to 2005. Using both multiple linear regressions and multiple logistic regression models, no statistically significant associations were found between PFOS and PFOA and sperm quality. Sperm quality in relation to serum PFAAs was also the major theme of a research project by *Toft et al. (2012)* conducted among 588 partners of pregnant women from Greenland, Poland and Ukraine. While semen volume and sperm count were unaffected by serum PFOS, PFOA, PFHxS and PFNA, the proportion *(95% CI)* of morphologically normal sperm cells were lower in the third tertile of PFOS (35%, 4% to 66%) and PFHxS (35%, 1% to 70%) exposure, relative to the first tertile. *Specht et al. (2012)* used the same cohort as *Toft et al. (2012)* but selected 604 fertile men for which serum levels of PFOS, PFOA, PFHxS and PFNA were available, and a battery of six reproductive hormones were assessed. No significant exposure-effect associations were reported. A third study on the same cohort of men from Greenland, Poland and Ukraine was conducted by *Kvist et al. (2012)* which tested the hypothesis that exposure to PFOS and PFOA can bias the

sperm sex chromosome ratio and thus skew the sex ratio in human populations. Using multiple linear regressions, a statistically significant negative association was detected between PFOS levels (categorized in quartiles) and sex ratio. The absence of adverse effect of PFAAs on semen quality was also an important finding of *Joensen et al. (2013)* as described in the previous section on endocrine disruption.

1.10.2.2.12 Adverse developmental outcomes

Since the publication of the first paper by *Hansen et al. (2001)* in 2001 documenting the presence of specific PFAAs at ng/mL in human serum, multiple studies have documented the maternal-fetal transfer of these contaminants in humans. Detailed reviews of these studies are given in the introductions of chapters 1 and 4. Based on strong and consistent scientific evidence that babies are exposed to a broad range of PFAAs in-utero, the next logical question is whether this pre-natal exposure can lead to adverse developmental outcomes. While in-vitro and in-vivo toxicology studies largely support this hypothesis, human epidemiology studies have not yet settled on the most prominent adverse outcomes and their severities.

One of the most studied end-points with respect to in-utero exposure to PFAAs is having low-birth weight (LBW) babies. *Inoue et al. (2004)* analysed 15 paired maternal and cord blood from delivering women in Japan and found statistically significant correlations between maternal and cord blood concentrations of PFOS (r^2 =0.876) , but not for PFOA. However, no correlations between either maternal or cord blood PFOS and birth weight was found. *Grice et al. (2007)* used

retrospective medical data of female employees in a PFOS manufacturing plant and birth weight of 439 singleton babies of these employees and reported no statistically significant association between PFOS exposure and birth weights. *Apelberg et al. (2007)* were the first to report on negative associations between multiple developmental endpoints and cord serum levels of PFOS and PFOA. Using multiple logistic regression, it was found that for each 2.7-fold increase in cord serum PFOS concentrations in fully adjusted models, the birth weight *(95% CI)* changed by -69 (-149 *to* 10) grams, the head circumference *(95% CI)* of the babies changed by -0.32 (-0.56 *to* -0.07) centimeters, body length changed by 0.13 (- 0.26 *to* 0.52) centimeters, and the ponderal index (units of measurement is g/cm^3 x 100) changed by -0.074 (-0.123 *to* - 0.025) units. For the same 2.7-fold increase in cord serum PFOA, the birth weight *(95% CI)* changed by -104 (- 213 *to* 5) grams, the head circumference *(95% CI)* of the babies changed by -0.41 (-*0.*76 *to* -0.07) centimeters, the body length changed by - 0.10 (-0.64 *to* 0.44) centimeters and the ponderal index (units of measurement is g/cm^3 x 100) changed by - 0.070 (- 0.138 *to* -0.001) units.

Fei et al. (2007) used data collected from 1,400 women and their babies from the Danish National Birth Cohort and tested the hypothesis that high maternal PFOS and PFOA were associated with LBW. While average maternal PFOS levels (35.3 g/mL) were higher than PFOA levels (5.6 ng/mL), only PFOA was significantly associated with birth weight with an adjusted β-value of -10.63 g; (*95% CI*, -20.79 *to* - 0.47).

Monroy et al. (2008) recruited 101 pregnant Canadian women to study the maternal-fetal transfer of PFAAs and reported that PFOS, PFOA, PFNA and PFHxS crossed the placental barrier with different transplacental transfer efficiencies (TTEs). As far their associations with LBW babies are concerned, multiple stepwise regression analysis controlling for confounders showed no evidence that individual PFAA adversely affected birth weights. Another hospital based prospective follow-up study conducted by *Washino et al. (2009)* on 423 Japanese women and their babies showed that birth weight was negatively correlated with maternal serum PFOS. For a 10-fold increase in maternal PFOS, the birth weight of the babies decreased by 148.8 g (*95% CI*: 0.5 *to* 297.0). When the statistical analysis was done after stratifying by gender of the babies, the association remained significant only for females babies with a decrease of 269.4 g (*95% CI*: 73.0 *to* 465.7) for each 10 fold increase in maternal PFOS concentrations. No such associations were found for PFOA.

Stein et al. (2009) conducted a population based cross-sectional study of exposure to PFOS and PFOA in a highly exposed community in the Mid-Ohio River Valley, with the outcome of interest being LBW. The authors reported a statistically significant association between maternal PFOS concentrations (above the median) and LBW with an odds ratio of 1.5 (*95% CI*: 1.1 *to* 1.9). A dose-dependent effect was even obtained when PFOS concentrations were transformed into a categorical variable, reaching an adjusted OR of 1.8 (*95% CI* 1.2 *to* 2.8) for PFOS levels above the 90^{th} percentile with respect to exposures below the 50^{th} percentile. *Nolan et al. (2009)* studied a similar highly exposed population in

Little Hocking, Washington County, Ohio, but used an ecological study design. Neonatal birth weight data on 1555 neonates were collected from the Ohio Department of Public Health and associations were made with Little Hocking Water Association category which was used as a surrogate of PFOA exposure. Multiple linear and logistic regressions were used to analyse the data and the authors reported no statistically significant associations between the predictor and outcome variables. Although the *Nolan et al. (2009)* study makes some contribution to knowledge on adverse birth outcomes to PFOA exposure, the study design used is prone to ecological fallacy whereby inference is made based on aggregate data across groups. *Hamm et al. (2010)* conducted a cross sectional study on 252 pregnant women from Alberta, Canada to verify the effect of maternal PFAA concentration on the birth weight of the babies. Multiple linear regressions show no evidence of any association between maternal PFOS, PFOA or PFHxS concentrations and LBW babies; for every 2.7 fold increase in the maternal serum concentrations of PFOS, PFOA and PFHxS the change in the babies' birth weight *(95% CI)* were 21.9 g (- 23.4 *to* 67.2), -37.4 g (- 86.0 *to* 11.2) and 31.3 g (- 43.3 *to* 105.9) respectively. *Chen et al. (2012)* recruited 429 mother-infant pairs from the Taiwanese Birth Panel Study to analyze for any possible associations between umbilical cord plasma levels of PFAAs and adverse birth outcomes. Adjusted β-coefficients *(95% CI)* of birthweight (in grams) for every $\log_e$increase (2.7-fold increase) for PFOS, PFOA and PFNA were -110.2 (-176.0 *to* -44.5), -19.2 (-63.5 *to* 25.1) and 6.07 (-16.6 *to* 28.7) respectively. Similarly, adjusted β-coefficients *(95% CI)* of head circumference (in cm) for every $\log_e$

increase (2.7-fold increase) for PFOS, PFOA and PFNA were -0.25 (-0.46 *to* -*0*.05), -0.05 (- 0.22 *to* -0.17), and 0.05 (-0.04 *to* 0.13) respectively. This study shows some consistency with *Stein et al. (2009)* and *Washino et al. (2009)* studies, such that significant associations were detected for PFOS but not for PFOA. *Lee et al. (2013)* conducted a cross-sectional study on 70 paired maternal and umbilical cord blood (UCB) sera obtained in South Korea, with PFAA concentrations as primary dependent variables and LBW as one of the outcomes of interest. Using multiple logistic regression, the authors reported a statistically significant inverse associations between (a) maternal PFOS concentration and ponderal index [OR=0.22 *95%CI*: 0.05 *to* 0.90)] and (b) UCB PFHxS concentration and birth weight [OR=0.26 *95%CI*: 0.08 *to* 0.85)]. This is the first study implicating PFHxS in a possible link to LBW. In a most recent Canadian study on 100 UCBs obtained from hospital deliveries in Ottawa, *Arbuckle et al. (2013)* reported weak associations (p=0.093) between UCB PFOA levels and low birth weight. Overall, although some of these studies found statistically significant associations between maternal and/or UCB serum PFOS and/or PFOA levels, the strength of these associations are not strong and there does not seem to be enough consistency across the published data to make causal inference today.

Adverse developmental outcomes subsequent to in-utero exposure may also occur later in life. A few studies looking at maternal exposure to PFAAs during pregnancy and adverse effects on the infants later in life have recently been published. *Christensen et al. (2011)* used a nested case-control study design to analyze data generated by the Avon Longitudinal Study of Parents and Children

(ALSPAC) in the United Kingdom, to examine any link between maternal PFAA levels during pregnancy and age at menarche of their daughters. In this study, 218 girls were classified as cases (menarche before the age of 11.5 yrs.) and 230 controls were selected among the remaining girls. While the PFCAs were associated with increased odds of early menarche, and the PFSAs were associated with decreased odds of early menarche, none of these associations were statistically significant at 95% confidence interval levels. *Maisonet et al. (2012)* used nearly the same ALSPAC cohort but performed cross-sectional analyses of the data using maternal levels of PFAAs as predictor variables, and weights at birth and at 20 months as the outcome variables. Tertiles of PFOS, PFOA and PFHxS exposures were made, and the first tertile for each analyte was used as the referent. Girls born to mothers in the 3rd tertile of exposure for all three PFAAs had statistically significant lower weights *(95% CI)* at birth compared to those in the 1st tertile; PFOS [-140 g (- 238 to - 42)], PFOA [-133 g (-237 *to* -30)], PFHxS [-108 g (- 206 *to* -10)]. Surprisingly, at 20 months of age, girls born to mothers in the 3rd tertile of PFOS exposure were 580 g (*95% CI*: 301 *to* 858) heavier compared to those in the 1st tertile. There were no differences in weights at 20 months across tertiles of PFOA and PFHxS exposure.

In the same context of prenatal exposure to PFAAs and adverse developmental outcomes, Fei and colleagues have published at least three papers with some variability in their findings depending on the end-point monitored. The first of these papers, published in 2008 *(Fei et al. 2008),* was based on a prospective follow-up of 1,400 mother-baby pairs in the Danish National Birth Cohort. The

exposure of interest was maternal serum PFOS and PFOA concentrations and outcomes were measured a three definite points in time: APGAR *(**A**ppearance, **P**ulse, **G**rimace, **A**ctivity and **R**espiration)* score shortly after birth, and maternally reported developmental milestones at 6 months and 18 months of age for the infants. Using the APGAR score as an ordinal variable, no significant association was found between PFOS and/or PFOA. When the latter was used as a dichotomous variable with an APGAR score of 10 being used as a cut-off value (*high score*: APGAR ≥10, *low score*: APGAR< 10), the odds *(95% CI)* of a low score when comparing the fourth quartile to the first quartile of maternal PFOS and PFOA exposure were 1.20 (0.67 *to* 2.14) and 1.14 (0.57 *to* 2.25) respectively. Using a structured questionnaire to assess multiple developmental milestones and their possible link to maternal prenatal PFOS and PFOA exposure, the authors found no "convincing" associations. *Fei and Olsen (2011)* conducted another study on the Danish National Birth Cohort to assess whether maternal serum PFOS and PFOA concentrations during pregnancy can potentially influence the behavior and motor coordination in childhood. When the children reached 7 years of age the mothers were asked to fill in a "*Strengths and Difficulties Questionnaire*" (SDQ, n=787) and a "*Developmental Coordination Disorder Questionnaire*" (DCDQ, n=526), based on which deciles of exposure were established. A child was categorized as having a behavioral problem if the SDQ score was >90th percentile and a developmental coordination disorder if the DCDQ score was <10th percentile. At median maternal serum PFOS and PFOA levels of 34.4 ng/mL and 5.4 ng/mL respectively, no significant associations were

found with behavioral and motor coordination problems in the children. The third study by *Fei et al. (2010),* which was discussed in the section "*Immune System Disorders*", looked at pre-natal exposure to PFOS and PFOA (maternal serum levels being used as surrogates) and the likelihood of the infants to be hospitalized for infectious diseases, and the data suggest no consistent association between the predictor and outcome variables of interest.

1.10.2.2.13 General comments on exposure-disease association studies

Although some of the above-mentioned studies found statistically significant associations between serum PFAAs (namely PFOS and PFOA) concentrations with various adverse health outcomes causality has not been proved in any of these them. Many of the studies where the purported associations are reported are based on cross-sectional data, whereby temporality cannot be controlled for. Furthermore, the strength of associations (odds ratios or relative risk) are quite low to have a meaningful biological effects and there is a definite lack of consistency of the statistical associations across different studies. Although there are multiple toxicological studies (Section 1.10.1) implicating PFOS and PFOA with adverse health effects in laboratory animals, it is hard to extrapolate these data to human populations to justify any biological plausibility, because firstly, the mechanism of disease in animals may be completely different in compared to humans and secondly, the exposure dose used in the toxicological studies are orders of magnitude higher than exposure levels in background human populations.

1.11 Knowledge gaps to be addressed in the thesis

As described, PFAAs may have important health consequences for exposed humans, but even for one of the most prominent PFAAs, PFOA, it is not clear if human exposure today is to historic ECF PFOA, or contemporary telomer PFOA. This is problematic because the presence of isomers in ECF PFOA could well affect the toxic response, yet most toxicological tests and all epidemiological surveys have ignored (i.e. not reported) the isomer profile of PFOA administered to animals, or measured in human subjects (i.e. only total PFOA is reported), respectively. Furthermore, although exposure pathway studies are consistent that food and dust are the primary exposure routes for PFOS and PFOA, little or no attention has been paid to the isomer profiles (and hence the manufacturing sources) in various human exposure media. Isomer profiling in environmental and human samples might, therefore, help to understand if humans are exposed to "old" or "new" sources of PFOA.

As stated earlier in the section "*Pre-natal exposure to PFAAs and adverse developmental outcome*s" there are dozens of studies to date documenting the presence of PFAAs in human cord blood. Thus, the scientific debate on whether or not PFASs can cross the placental barrier is no longer a leading question. However, significant knowledge gaps still exist on the maternal-fetal transfer. First, as a result of historical production of PFOS and PFOA by electrochemical fluorination, humans are exposed to a mixture of linear and branched PFOS and PFOA, and it is unknown which isomers are more efficient at crossing the placental barrier. If there is a differential efficiency of linear and branched

isomers at crossing the placental barrier, then the exposure scenario for the fetus may be completely different from that of the mother and from adults in general. Thus, it is important to shed light on the isomer specific maternal-fetal transfer of PFAAs, and moreover whether transfer of PFAAs across the human placental barrier is also chain length dependent.

As described, it is established that PFAAs cross the human placental barrier, and that renal excretion may be an important mode of clearance in all people. Both of these processes will be affected by the extent of PFAA protein binding in serum, yet there are currently no isomer specific data for any PFAA, including dissociation constants (K_d's). All of these unknowns, and others, are explored through monitoring, biomonitoring, and experimental work in the next four experimental chapters.

This thesis has five broad objectives. These are listed below, followed by the working hypothesis for each:

1. To elucidate whether present day human exposure via house dust is to currently manufactured (telomer) PFOA (almost exclusive linear PFOA) or historically manufactured PFOA (mixture of branched and linear PFOA) (Chapter 2).

   Hypothesis: Humans are primarily exposed to telomer PFOA

2. To verify whether linear isomers of PFOS and PFOA cross the human placental barrier more efficiently than the respective branched isomers. (Chapter 2)

   Hypothesis: Linear PFOS and PFOA will have higher transplacental transfer efficiencies than their branched counterparts.

3. To track the source and pathway of exposure to PFHxS in a Canadian family having exceptionaly high serum levels of PFHxS. (Chapter 3)

   Hypothesis: The source of PFHxS in the family is the house carpet and the pathway of exposure is likely through the inhalation or ingestion of contaminated house dust (Chapter 4).

4. To compare the binding affinities of linear and branched isomers of PFOS and PFOA to human serum albumin (Chapter 5).

   Hypothesis: Linear isomers of PFOS and PFOA should have lower dissociation constants (i.e. stronger binding) with human serum albumin compared to the branched isomers.

5. To compare the transport efficiencies of linear and branched isomers of PFOS and PFOA across a monolayer of human placental trophoblast cells in an in-vitro model of the placenta.

   Hypothesis: Linear isomers of PFOS and PFOA should cross the monolayer trophoblast cell layer more efficiently than the respective branched isomers.

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CHAPTER 2: Isomer Profiles of Perfluorochemicals in Matched Maternal, Cord and House Dust Samples; Manufacturing Sources and Transplacental Transfer

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Abstract

Perfluorochemicals are detectable in the general population and in the human environment, including house dust. Sources are not well-characterized, but isomer patterns should enable differentiation of historical and contemporary manufacturing sources. Isomer-specific maternal-fetal transfer of perfluorochemicals has not been examined despite known rodent developmental toxicity of perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA). To elucidate relative contributions of electrochemical (phased out in 2001) and telomer (contemporary) perfluorochemicals in dust, and to measure how transplacental transfer efficiency (TTE, based on a comparison of maternal and cord sera concentrations) is affected by perfluorinated chain-length and isomer branching pattern. Matching samples of house dust (n=18), maternal sera (n=20) and umbilical cord-sera (n=20) were analyzed by isomer specific HPLC-MS/MS.

PFOA isomer signatures revealed that telomer sources accounted for 0 to 95% of total PFOA in house dust (median = 31 %). This may partly explain why serum PFOA concentrations are not declining in some countries despite the phase-out of electrochemical PFOA. TTE data indicate that total branched isomers crossed the placenta more efficiently than linear isomers for both PFOS ($p<0.01$) and PFOA ($p=0.02$), and that placental transfer of branched isomers of PFOS increased as the branching point moved closer to the sulfonate (SO_3^-) end of the molecule. Results suggest that humans are exposed to telomer PFOA, but larger studies that also account for dietary sources should be conducted. The exposure profile of PFOS and PFOA isomers can differ between the mother and fetus, an important consideration for perinatal epidemiology studies of perfluorochemicals.

2.1 Introduction

The most prominent perfluorochemicals (PFCs) in human samples are perfluorooctane sulfonate (PFOS), perfluorooctanoate (PFOA), and perfluorohexane sulfonate (PFHxS), yet the sources and pathways of human exposure to these, and other PFCs, are not well-characterized. Perfluorinated acids are ubiquitous in the global environment, owing to their long history of manufacture and resistance to biological and environmental degradation pathways. Specifically for PFOA, the manufacturing sources responsible for its presence in various environments are not understood, and future human exposure is therefore difficult to predict. There are 2 main manufacturing methods leading to PFOS and PFOA, electrochemical fluorination (ECF) and telomerization. The 3M Co. manufactured the bulk of PFOS (and higher molecular weight precursor

materials), PFHxS, and PFOA by ECF until 2001, at which time they voluntarily phased-out these chemistries. Nonetheless, PFOS and its precursors continue to be manufactured by other companies in Asia *(Martin et al. 2010)*. Telomerization continues to be used to manufacture PFOA. ECF and telomerized PFOA can be readily distinguished analytically because ECF PFOA consists of a mix of linear and branched isomers *(Loveless et al. 2006, Reagen et al. 2007)*, while telomerized PFOA is almost exclusively the linear isomer *(Kissa 1994*).

If humans are predominantly exposed to ECF sources of PFOS and PFOA, serum concentrations should be decreasing due to their phase-out. In fact, when the 3M Co. stopped manufacturing PFOS and PFOA by its ECF technique, blood levels of PFOS declined steadily in Americans. However, for PFOA the initial rate of decline was much less than anticipated *(Olsen et al. 2008)*, and the most recent data from the National Center of Health Statistics of the US Centers for Disease Control and Prevention *(Kato et al. 2011)* indicate that serum PFOA did not decline between 2003/04 and 2007/08, and may be increasing (Figure 2.1). This suggests that exposure to recently-produced telomer sources of PFOA might be important, but the relative importance of ECF- and telomer-derived PFC exposures through different exposure pathways (diet, dust, water, air, etc.) is unknown. Nonetheless, the potential for telomer PFOA exposure is recognized, and in 2006 a global stewardship program was implemented to reduce emissions of this chemical (U.S. EPA 2006).

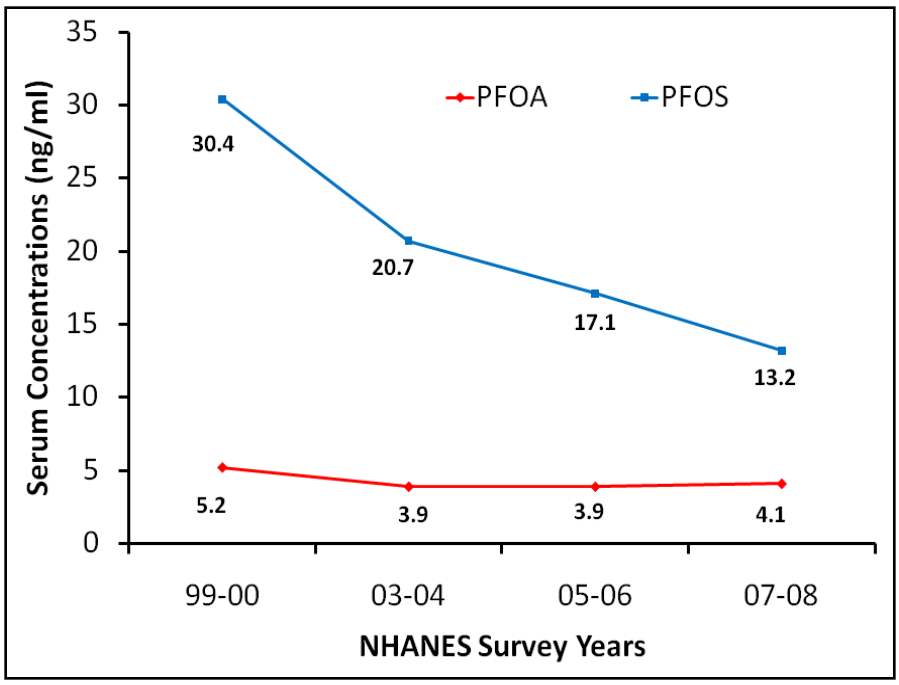

Figure 2.1. Trend in the geometric mean concentrations of PFOA and PFOS in the blood of Americans for the 10 year period from 1999 to 2008. PFOS has consistently declined, whereas PFOA has not declined since 2003/2004 and may be increasing. Data for the first 3 NHANES surveys are published data by the CDC, while the geometric means for 2007-2008 were calculated (after weight adjustment) using SAS data files available on the website of the National Center for Health Statistics of the CDC.

For many environmental chemicals, house dust can be a major source of exposure *(Butte et al. 2002)*, particularly for children *(US EPA 2008).* For PFOS and PFOA, food is a major source of exposure, but house dust can also be important under scenarios of high dust-ingestion *(Tittlemeir et al. 2007, Bjorklund et al. 2009, Goosey and Harrad 2011, Shoeib et al. 2011, Haug et al. 2011).* Thus, it is important from a risk mitigation perspective to understand whether PFCs in house dust are from current or historical manufacturing sources. PFOS and PFOA have been measured in dust previously *(Kato et al. 2009, Kato et al. 2009, Kubwabo et al. 2005)*, but isomer specific PFC analytical methods *(Benskin*

et al. 2007; Langlois and Oehme 2006) have not been used to determine the manufacturing origins of PFOA and other PFCs in house dust.

Loveless et al. (2006) demonstrated that linear ammonium PFOA was generally more toxic than branched PFOA, but the isomer specific toxicity of PFCs have not been examined because of the lack of available standards. Studies in rats and zebrafish show that PFOA and PFOS are developmental toxicants *(Lau et al. 2004)*, and many human epidemiology studies are now emerging on the potential perinatal effects of PFCs. For example, some epidemiology studies have shown inverse associations between PFOA exposure and birth weight (*Apelberg et al. 2007; Fei et al. 2008)*, while others did not find any association (Monroy et al. 2008; Nolan et al. 2009). Furthermore, other adverse human health effects associated with PFCs are being detected in both background *(Nelson et al. 2010)* and highly exposed populations *(Steenland et al. 2010).* From a public health perspective, and recognizing that many PFCs occur as multiple isomers of unknown relative toxicity, it may be important to characterize the exact nature of PFC exposure to humans, including for the mother and the fetus.

Understanding the maternal-fetal transmission of PFCs is necessary to clearly understand the risks and mechanisms of human developmental toxicity. Of studies that have reported the maternal-fetal transfer of PFCs, *Hanssen et al. (2010)* was the only study to examine branched isomers separately from linear isomers. However, individual branched isomers were not examined separately (i.e. total branched PFOS was compared to linear PFOS). Although we are beginning to understand the pharmacokinetic properties of specific branched

PFCs in animal models *(De Silva et al. 2009)*, no study has yet investigated isomer-specific PFC pharmacokinetics in humans. In an attempt to understand the transplacental transfer of PFCs (mainly PFOS and PFOA), multiple studies have tested maternal and umbilical cord blood samples from different populations *(Needham et al. 2011, Kim et al. 2011, Fromme et al. 2010, Hanssen et al. 2010, Monroy et al. 2008, Midasch et al. 2008, Fei et al. 2007, Inoue et al. 2004).* One consistent finding in all these studies is that cord serum has lower total PFOA and lower total PFOS than maternal serum; however the isomer-specific transplacental transfer of the various branched isomers has not been examined despite evidence that the placental transfer of total branched PFOS isomers is greater than for linear PFOS *(Hanssen et al. 2010).*

In the current work we collected dust from the homes of twenty pregnant women who also donated a blood sample at 15 weeks of gestation and a cord blood sample at delivery. We measured PFC concentrations and isomer profiles in all samples in an effort to identify sources of PFCs in house dust, and to examine the isomer specific transfer of PFCs across the placenta.

2.2 Materials and Methods

2.2.1 Nomenclature and Acronyms.

For structural isomers, we use the nomenclature defined by Benskin et al. (2007). Using PFOS as an example, the following annotations are used to represent the structure of each isomer based on relative position of perfluoromethyl substitution: linear perfluorooctane sulfonate (*n*-PFOS), perfluoroisopropyl (*iso*-PFOS), 5-perfluoromethyl (5*m*-PFOS), 4-perfluoromethyl (4*m*-PFOS), 3-

perfluoromethyl (3*m*-PFOS), 1-perfluoromethyl (1*m*-PFOS), tert-perfluorobutyl (*tb*-PFOS) and sum of all dimethyl isomers (Σm_2-PFOS). With the exception of *n*-PFOS, all of the above mentioned isomers are branched isomers. (For structures see Figure 2.2).

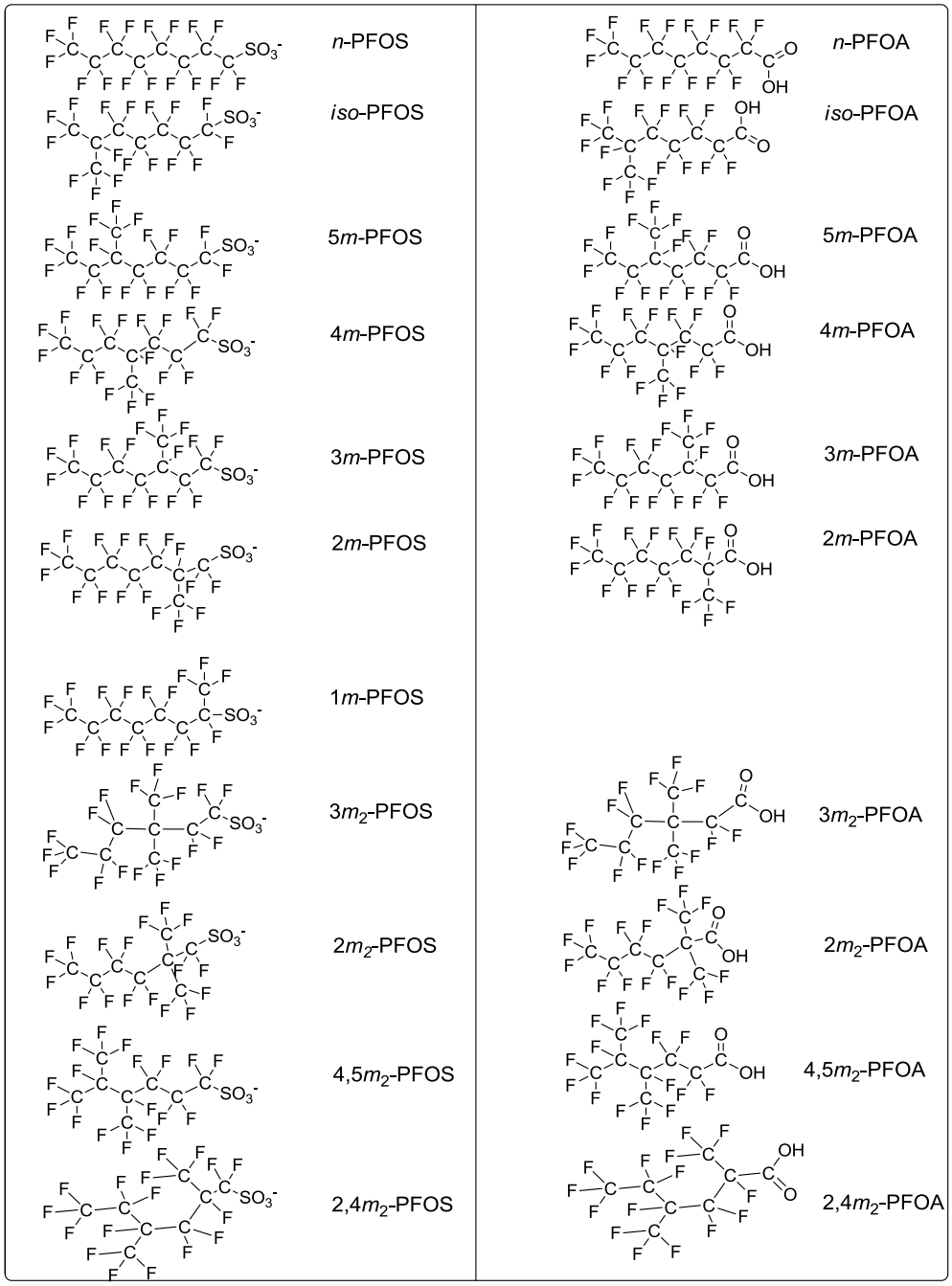

Figure 2.2 Structures of PFOS and PFOA linear and branched isomers. Note that 1m-PFOA does not exist. For geminal diperfluoromethyl-PFOS and geminal diperfluoromethyl-PFOA isomers, only the 3m2 and 2m2 are shown, although other such structures exist. Similarly for non-geminal diperfluoromethyl PFOS and PFOA only the 4,5 m2 and 2,4 m2 are shown, but other structures exist. In the isomer specific analysis, all the diperfluoromethyl PFOS isomers are grouped together and labeled as Σm2-PFOS. Other nomenclature systems have been proposed (Rayne et al. 2008).

2.2.2 PFC Chemical Standards.

The 3M Co. donated ECF PFOS (30 % branched and 70 % linear, by ^{19}F NMR) and PFOA (22 % branched and 78 % linear, by ^{19}F NMR) standards (Reagen et al. 2007). All other PFC standards, including PFOS and PFOA isomer standards, and linear mass-labeled internal standards for PFBA, perfluorohexanoate (PFHxA), PFHxS, PFOA, perfluorononanoate (PFNA), PFOS, perfluorodecanoate (PFDA), perfluoroundecanoate (PFUnA) and perfluorododecanoate (PFDoA) (MPFAC-MXA), were obtained from Wellington Laboratories (Guelph, ON, Canada).

2.2.3 Blood Collection.

Samples (n=20) analyzed in this study are a subset of participants recruited in 2007-2008 to a Canadian (Vancouver, BC) cohort study named Chemicals, Health and Pregnancy (CHirP) *(Webster et al. 2011).* All participants provided informed consent. Laboratory personnel collected blood samples from pregnant volunteers in Vancouver, BC, at 15 weeks of gestation, and samples of cord blood (n=20) were collected at delivery. After serum separation, all samples were stored at -80 °C. Ethical clearance was obtained from the Research Ethics Boards of the University of British Columbia, the University of Alberta, Health Canada, and the 3 participating hospitals.

2.2.4 Serum Preparation.

The method of *Kuklenyik et al. (2004)* was adapted for the extraction of PFCs from serum using a Rapid Trace system (Caliper Life Sciences, Hopkinton, MA). The solid phase extraction cartridge (Oasis-HLB, Waters, 60 mg/3mL) was

conditioned with 2 mL of methanol followed by 2 mL of 0.1 M formic acid. Serum was prepared for extraction by mixing 3 mL of 0.1 M formic acid with 1.0 mL of serum. Following the addition of mass labelled internal standards (10 ng of each), the mixture was vortexed and sonicated for 20 min. Prepared serum was added to the column and washed successively with 3 mL of 0.1 M formic acid, 6 mL of 50 % 0.1M formic acid/50 % methanol, and 1 mL of 1 % ammonium hydroxide. The cartridge was drained by vacuum and PFCs were eluted with 1.0 mL of 1 % ammonium hydroxide in acetonitrile. The eluate was concentrated to 100 µL followed by the addition of 200 µL of 90 % 20 mM acetic acid in 10 % methanol. Method blanks containing calf serum, a calf serum sample spiked at 0.5 ng/mL of each PFC, and a human serum sample spiked at 10 ng/mL were analyzed with the real samples.

2.2.5 Dust Collection and Extraction.

Dust samples were also a subset of those collected for the larger CHirP study (Shoeib et al. 2011). At 20-24 weeks of gestation, participants donated a used vacuum cleaner bag from their vacuum cleaner, or we took grab samples from the participants' bagless vacuum cleaners (n=18). Samples were stored at -20 °C, and before analysis a portion of each dust sample was sieved using a stainless steel sieve (mesh size 150 µm, VWR International, Montreal, Quebec). Sieved dust (0.1 g) was spiked with 3.3 ng of mass labeled internal standards (MPFAC-MXA), and 4 mL of methanol was added and vortexed for 5 min, sonicated for 1 hr., and centrifuged at 3400 rpm for 10 min. A 2 mL aliquot was reduced by

evaporation to 100 µL, and 200 µL of 20 mM acetic acid with 10 % methanol was added before HPLC-MS/MS.

2.2.6 Total PFC Analysis.

For total PFC concentrations, separation was by HPLC on a 150 mm Synergi™ Hydro-RP C-18 column (Phenomenex, Torrance, California). Gradient elution at 600 µL/min used A (20 mM ammonium acetate (pH 4) in water) and B (methanol) mobile phases. Initial conditions were 60 % A for 1 min, ramped to 20 % A by 3 min followed by a 5 min hold and a subsequent increase to 100 % B by 8.5 min, held until 14 min at which time initial conditions were re-established. Tandem mass spectrometry (MS/MS) data were collected on an Applied Biosystems API 3000 (Carlsbad, California), using electrospray ionization in negative ion mode. For mean recoveries of total PFCs in serum and dust, see Table 2.1.

Table 2.1. Spike and recovery of native PFCs in serum and dust. Experiments were done in triplicate in each case and figures presented here represent arithmetic means.

PFCs	Calf serum spiked at 0.5 ng/ml		Calf serum spiked at 10 ng/ml		Dust spiked at 60 ng/g	
	Mean % Recovery ±S.D	Range	Mean % Recovery ±S.D	Range	Mean % Recovery ± S.D	Range
PFBS	93.2 ± 16.9	79.4-128	-	-	71.7 ± 8.1	64.0 - 83.0
PFHxS	79.0 ± 13.0	61.8-94.8	91.8 ± 12.5	75 – 112	90.2 ± 12	72.1 - 105
PFHpS	95.5 ± 15.1	76.8-123	-	-	92.3 ± 27	46.8 - 116
PFOS	82.7 ± 16.8	52.0-99.3	93.1 ± 11.9	82 – 117	107 ± 14	89.9 - 121
PFDS	109 ± 8.7	93.3-120	64.0 ± 8.2	52 – 74	32.2 ± 15	14.7 - 48.9
PFBA	-	-	-	-	74.3 ± 8.9	60.1 - 84.7
PFPeA	-	-	-	-	83.7 ± 11	65.7 - 95.0
PFHxA	94.2 ± 9.3	78.3-108	-	-	128 ± 9	119 - 141
PFHpA	78.1 ± 10.7	66.2-97.4	-	-	97.9 ± 21	72.6 - 130
PFOA	82.5 ± 9.4	67.1-99.1	95.3 ± 15.5	68 – 129	83.0 ± 5.3	74.6 - 88.5
PFNA	94.4 ± 5.4	84.7-102	94.8 ± 8.3	74 – 101	93.1 ± 20	72.0 - 117
PFDA	81.9 ± 7.6	71.6-93.8	96.9 ± 4.6	91 – 106	102 ± 9	92.1 - 112
PFUnA	92.8 ± 7.6	81.6-106	93.8 ± 10.4	78 – 116	107 ± 11	95.1 - 118
PFDoA	102 ± 4.9	97.3-113	93.3 ± 5.8	83 – 101	95.6 ± 6.3	91.3 - 106
PFTrA	122 ± 6.9	115.0-133	54.0 ± 11.0	33 - 70	79.2 ± 17	59.9 - 106
PFTA	105 ± 7.4	97.2-117	-	-	42.1 ± 24	10.2 - 76.3
FOSA-M	108 ± 7.6	92.3-116	-	-	42.7 ± 4.9	38.2 - 50.9

2.2.7 Isomer Specific PFC Analysis.

The isomer-specific HPLC-MS/MS method was adapted from Benskin et al. (2007). Briefly, 3 µL of the same extracts analyzed for total PFCs were injected

onto a FluoroSep RP Octyl column (ES Industries, West Berlin, NJ). Flow rate was 200 μL/min, and starting conditions were 60 % A (water adjusted to pH 4.0 with ammonium formate) and 40 % B (methanol). Initial conditions were held for 0.3 min, ramped to 64 % B by 1.9 min, increased to 66 % B by 5.9 min, 70 % B by 7.9 min, 78 % B by 40 min, 88 % B by 42 min and finally to 100 % B by 45 min and held until 60 min. Mass spectral data were collected using a 5000Q mass spectrometer (MDS Sciex, Concord, ON, Canada) equipped with an electrospray interface operating in negative ion mode. Chromatograms were recorded by multiple reaction monitoring (MRM) with 3 to 13 transitions per analyte.

2.2.8 Quality Control.

Triplicate recovery experiments were performed at 2 concentrations of native linear standards spiked to calf serum or dust (Table 2.1). There are no mass-labeled internal standards for branched PFOS or PFOA isomers, thus a standard addition experiment was done in dust to rule out possible matrix effects on the measured isomer profiles. Additionally, a vacuuming experiment was done to check if off-gassing during vacuuming may bias the dust isomer profile. The results of these two experiments clearly showed that matrix effects and off-gassing during vacuuming were not a problem. The % recovery during serum extraction was similar for all PFOS and PFOA isomers, such that the extraction step had no effect on the resulting isomer profiles (*Benskin et al. 2007*).

2.3 Results and Discussion

2.3.1 Total PFC Concentrations in House Dust.

All total PFCs, except for PFDS, were log-normally distributed (Shapiro-Wilk test, see Table 2.2 for distributions). The three major PFCs in all dust samples (n=18) were PFOA, PFOS, and PFHxA, with similar median values of 38, 37 and 35 ng/g, respectively. However, PFHxS exceeded PFOS in 4 samples.

This pattern, whereby PFOA, PFOS, and PFHxA were the dominant PFCs, is not dissimilar from *Strynar and Lindstrom (2008)* who monitored US house dust collected in 2001/2002 and found median PFOA, PFOS and PFHxA concentrations of 142, 201, and 54.2 ng/g, respectively. The higher concentrations of PFOS and PFOA observed by *Strynar and Lindstrom (2008)* are understandable given that these dust samples were collected years earlier than in the current study, around the time of the phase-out of ECF C8 chemistries, though sampling strategy and geography may also have contributed to differences.

Table 2.2. Summary statistics of total PFC concentrations (ng/g, non-isomer specific) in house dust samples (n=18 unless otherwise noted). To compute the descriptive statistics, values less than limit of detection (LOD) have been replaced by LOD/2.

	Min	Max	Median	Mean	Geometric Mean	% above LOD
Perfluoroalkyl sulfonates						
PFBS	<0.5	48	<0.5	6.1	0.7	28
PFHxS	2.9	1300	14	140	21	100
PFHpS	<0.5	46	<0.5	4.1	0.6	22
PFOS	<0.5	1300	37	180	39	94
PFDS	<0.5	5.1	2.1	2.2	1.8	94
Perfluoroalkyl carboxylates						
PFBA	<0.5	42	2.6	9.2	3.6	94
PFPeA	<0.5	93	5.2	17	4.9	83
PFHxA	2.3	390	35	77	33	100
PFHpA	1.4	320	21	55	19	100
PFOA	4.3	820	38	120	50	100
PFNA	1.4	220	15	44	18	100
PFDA	1.7	250	15	44	16	100
PFUA	<0.5	240	6.1	31	8.0	94
PFDoA	1.4	160	10	36	13	100
PFTrA	<0.5	67	2.4	9.9	2.3	78
PFTA	<0.5	24	3.3	6.5	3.3	94
Perfluoroalkyl sulfonamides						
PFOSA	<0.5	<0.5	<0.5	<0.5	0.3	0
NMeFOSA (n=16)	1.2	13.8	2.3	3.0	2.5	100
NEtFOSA (n=16)	<0.06	2.8	0.15	0.55	0.14	50
NMeFOSAA	<0.5	440	1.2	36	2.3	50
NEtFOSAA	3.2	240	27	58	32	100
NMeFOSE (n=16)	15	910	49	152	65	100
NEtFOSE (n=16)	<0.02	190	10	14	5.3	88

2.3.2 PFC Isomer Profiles in House Dust.

For PFOS, six major branched isomers were detected in dust: 1*m*-, 3*m*-, 4*m*-, 5*m*-, *iso*-, and Σm_2-PFOS. All the dust samples had PFOS isomer profiles that were very similar to the 3M Co. ECF standard of PFOS, with a mean branched isomer content (±SD) of 30±2.7 %, and relatively low variability among individual branched isomers in various samples (Figure 2.3A and Table 2.3). This was not surprising given that the 3M Co. produced the bulk of PFOS (Paul et al. 2009),

and that the historical batch to batch variation of branched isomer content was small: 30±0.8 % branched PFOS in eighteen lots over 20 years (Reagen et al. 2007).

Unlike PFOS, PFOA isomer profiles in dust were often substantially different from the 3M ECF PFOA standard. Although the relative profile among individual branched PFOA isomers was consistent among dust samples (Figure 2.3B and Table 2.3), there was an excess signal of linear PFOA in many of the samples compared to the 3M Co. ECF standard. Like 3M Co. PFOS, batches of 3M Co. PFOA also had a consistent isomer composition: 22±1.2 % branched isomers in 18 lots over 20 years *(Reagen et al. 2007).*

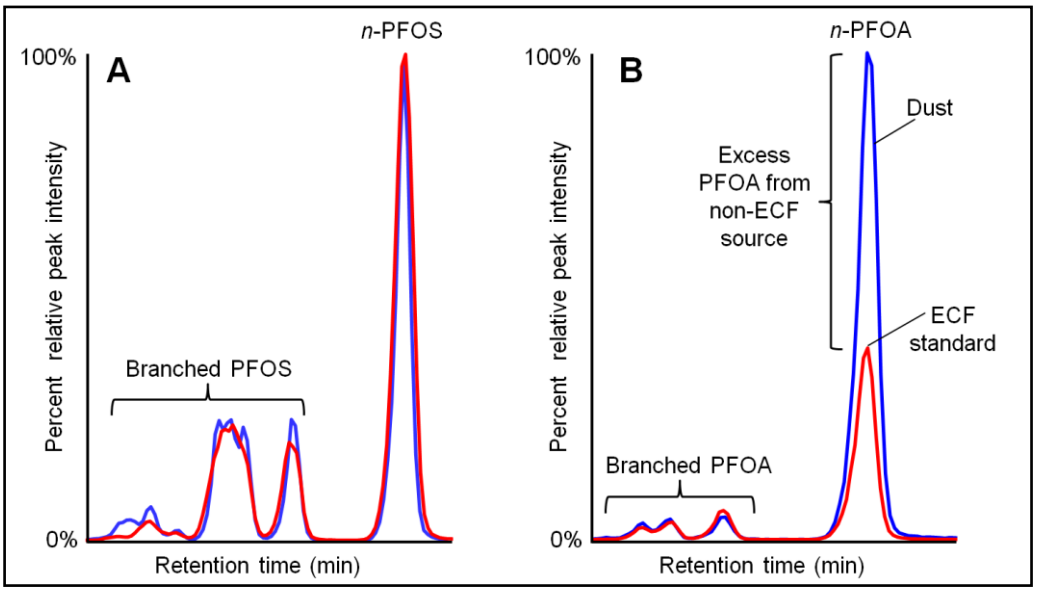

Figure 2.3. Isomer profiles in a house dust sample for (A) PFOS (m/z 499/80) and (B) PFOA (m/z 413/369). Red traces represent the isomer profile in a 3M ECF standard, while the blue trace represents the isomer profile in a dust sample. Each profile was normalized to the response of the branched isomers, such that the relative amount of linear isomer in each case is easily compared

Therefore, these observations suggest that a significant proportion of PFOA in these house dust samples came from a manufacturing source that used telomerization instead of ECF. The "% telomer" PFOA in each dust sample was calculated from the excess signal of linear isomer in samples (*m/z* 413/369 transition), compared to 3M Co. ECF PFOA. The % telomer ranged from 0 to 95% with a median of 31% among all samples (Figure 2.4). The presence of telomer PFOA in the human household environment may partly explain why total PFOA in serum has declined so slowly following the phase out of ECF perfluorooctyl chemistries by 3M Co. in the US (*Olsen et al. 2008*, Figure 2.1). However, telomer PFOA also may be present in food, and telomer PFOA-precursors used in food packaging may be absorbed and biotransformed to PFOA after ingestion *(D'eon and Mabury 2011).*

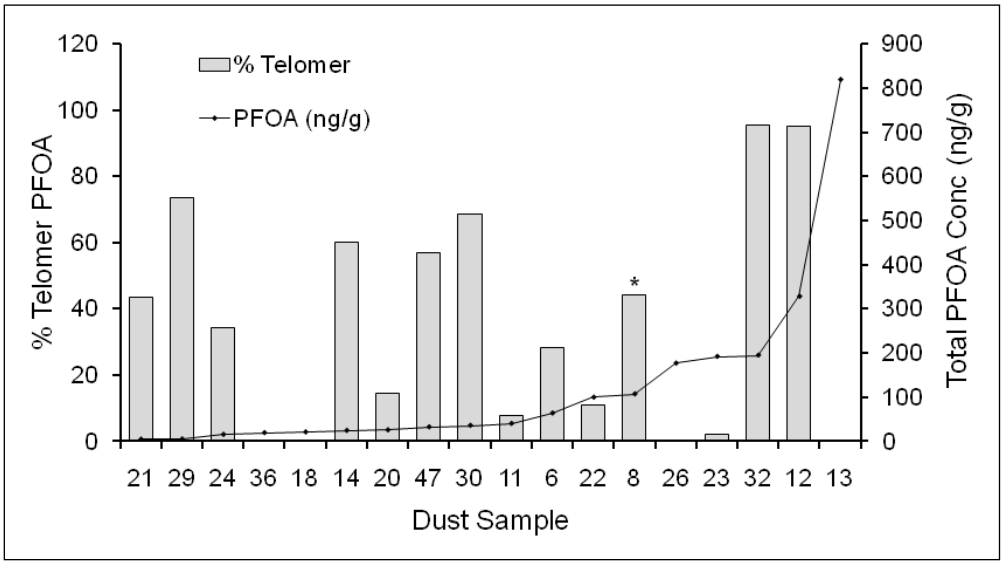

Figure 2.4. Percent telomer PFOA (grey bars) and total PFOA concentration (ng/g, black line) in house dust samples. Samples are shown from lowest to highest total PFOA concentration, from left to right. * indicates a sample collected by mechanical sweeper, instead of vacuum.

Isomer-specific chromatograms of house dust clearly indicated that other perfluorocarboxylates (i.e., PFCs with $-CO_2^-$ as a functional group) had only minor branched isomer content (data not shown). Authentic standards were not available for confirmation, thus we identified peaks as branched isomers only when 2 characteristic MS/MS transitions responded at the same retention time. Most perfluorocarboxylates other than PFOA appeared exclusively linear, but in a minority of dust samples PFNA had up to 4 minor branched isomers, while PFHxA, PFDA, PFUnA and PFDoA each had up to 2 minor branched isomers. The manufacturing sources of these particular branched perfluorocarboxylates cannot be confirmed due to limited information on their manufacturing sources and a lack of reference materials, but they may be residuals from ECF manufacturing of PFOS and PFOA. Perfluorosulfonates, such as PFDS, perfluoroheptane sulfonate (PFHpS), and PFHxS, are generally assumed to have been produced exclusively by ECF, and these all had major branched isomer content based on peak areas (data not shown). However, because reference materials were not available, we could not examine how closely they resembled authentic ECF manufacturing sources.

Table 2.3. Descriptive statistics of the percentage of individual isomers of PFOS and PFOA in house dust samples (N=18), and mean

	PFOS							**PFOA**						
	Linear	*iso*	5*m*	4*m*	3*m*	1*m*	$\sum m_2$	Linear	*iso*	5*m*	4*m*	3*m*	$\sum m_2$	*tb*
Mean	69.4	17.6	4.55	0.61	5.08	0.81	2.13	82.0	5.54	4.77	3.12	3.21	0.80	0.56
SD[a]	4.81	3.60	1.01	0.16	1.78	0.77	0.68	11.2	3.28	3.57	3.01	1.77	0.59	0.4
Median	70.7	16.2	4.33	0.58	5.1	0.58	2.00	84.2	4.99	4.21	2.69	3.37	0.68	0.55
Min	53.4	14.8	3.20	0.42	<LOD[b]	<LOD	1.34	59.0	0.46	<LOD	<LOD	0.19	0.05	0.03
Max	73.5	27.6	7.73	1.14	9.65	2.26	4.31	98.6	12.2	11.6	8.15	6.17	2.38	1.62
Mean ECF Std	70.2	18.1	4.83	0.58	4.18	0.46	1.69	73.4	9.05	7.35	5.80	2.98	0.74	0.67

(n=3 injections) of the 3M ECF standard.

[a] The low relative standard deviations (SDs) associated with the mean percentage of PFOS isomers in the 18 dust samples suggest a similar ECF source, contrasted to the high SD for linear PFOA, suggesting a mixture of ECF and telomer sources.

[b] LOD-Limit of detection.

2.3.3 Total PFCs in Maternal and Cord Sera.

Concentrations of total PFOS (n=20), PFOA (n=20), PFNA (n=20), PFDA (n=16) and PFHxS (n=8) in maternal serum were always significantly higher ($p<0.01$) than in cord serum, consistent with previous maternal-fetal transfer studies of PFOS and PFOA (Table 2.4).

The major total PFCs in maternal and cord sera were PFOS, PFOA, PFHxS and PFNA, similar to previous findings *(Inoue et al. 2004; Midasch et al. 2007; Monroy et al. 2008).* In the current study, the mean concentrations of PFOS, PFOA, PFHxS, PFNA and PFDA in maternal serum (and cord serum) were 5.5 (1.8), 1.8 (1.1), 1.7 (0.7), 0.9 (0.4) and 0.4 (0.1) ng/mL respectively. PFUnA, PFDoA and perfluorotetradecanoate (PFTA) were detected (detection limit 0.1 ng/mL) in 6, 2, and 3 maternal samples, respectively, but were not detected in any cord samples.

Table 2.4. Summary of existing studies on maternal-fetal transfer of total PFOA and total PFOS.

Study	Sampling year	Location	Sample Size	Mean Ratio of Cord to Maternal Serum Concentration (Corr. Coefficient)	
				PFOA	PFOS
Needham et al. (2011)	2000	Faroe Islands	12	0.72 (0.91)[a]	0.34 (0.82) [a]
Kim et al. (2011)	2007	Korea	20	0.69 (0.88) [a]	0.36 (0.50)[b]
Fromme et al. (2010)	2007-2009	Germany	27	0.70 (0.94)[b]	0.30 (0.89)[b]
Hanssen et al. (2010)	2005-2006	South Africa	58	0.71 (0.67)[b]	0.45 (0.88)[b]
Monroy et al. (2008)	2004-2005	Canada	101	0.81 (0.88) [a]	0.45 (0.83) [a]
Midasch et al. (2008)	2003	Germany	11	1.26 (0.72)[b]	0.60 (0.42)[b]
Fei et al. (2007)	1996-2002	Denmark	50[c] 50[d]	0.55 0.68 (0.84)[a]	0.29 0.34 (0.72)[a]
Inoue et al. (2004)	2003	Japan	15		0.32 (0.88)[a]
Current Study	2007	Canada	20[d] 20[e]	0.61 (0.63)[b] 0.71 (0.76)[e]	0.33 (0.81)[b] 0.36 (0.81)[e]

[a] Pearson Correlation, [b] Spearman Rank Correlation, [c] maternal serum was sampled in the 1st trimester, [d] maternal serum was sampled in the 2nd trimester, [e] TTE adjusted from 15 weeks to time of delivery (approximately 40 weeks) using data of Monroy et al. (2008) whereby PFOS declined 10 % and PFOA declined 12 % between the 24th-28th week and delivery.

Transplacental transfer efficiencies (TTEs) were estimated by dividing the PFC concentrations in cord serum at delivery by maternal serum concentration at 15 weeks gestation for each mother-cord pair (Table 2.5). Mean TTEs were always below 1.0, indicating lower concentrations in the cord serum than maternal serum (all $p<0.01$). Overall, the PFOS and PFOA TTEs were within the range reported in the literature. However, it is likely that our TTEs slightly underestimate actual TTE values because they do not reflect hematologic changes that occur later in pregnancy, including expansion of total plasma volume *(Whittaker et al. 1996).* Such an effect was shown by *Monroy et al. (2008)* who reported lower serum PFOS and PFOA levels in maternal serum samples collected at delivery versus 24^{th}-28^{th} weeks of gestation, and by *Fei et al. (2007)* who reported higher cord-maternal ratios based on maternal serum samples collected during the 2^{nd} trimester than the 1^{st} trimester (Table 2.4). We used the above data from *Monroy et al. (2008)* (see Table 2.4, footnote e) to estimate time-of-delivery maternal serum concentrations, based on our 15-week data, but this had little effect on the resulting TTE, and both adjusted and unadjusted TTE values were within the range of TTEs reported previously (Table 2.4).

A comparison of TTE among the three major perfluoroalkyl carboxylates (PFOA, PFNA, and PFDA) suggests that the longer-chain carboxylates were more efficiently blocked by the placental barrier (Figure 2.4A), consistent with the results of *Kim et al. (2011).* The same trend was also evident for the two major perfluorosulfonates (PFHxS and PFOS, Figure 2.4B). Overall, shorter-chained

PFCs crossed the placenta more efficiently than longer chain PFCs, consistent with the findings of *Needham et al. (2011).*

2.3.4 PFC Isomer Profiles in Maternal and Cord Sera.

The % branched content of total PFOS was consistently and significantly higher in cord serum than in corresponding maternal serum and dust samples (Figure 2.6). Branched PFOS isomers contributed 27- 44 % (median 36 %) of total PFOS in maternal serum, and from 36-54 % (median 46 %) in cord serum. A paired t-test indicated statistically greater proportions of branched PFOS in the cord serum ($p<0.01$). Overall, all branched PFOS isomers were transferred more efficiently (median TTEs of the different branched isomers = 0.34 - 0.88) than the linear isomer (median TTE = 0.30) (Table 2.5). This is similar to *Hanssen et al. (2010),* wherein a statistically greater relative abundance of linear PFOS was reported in maternal serum than cord serum, relative to total branched PFOS isomers ($p<0.05$ by Wilcoxon's signed rank).

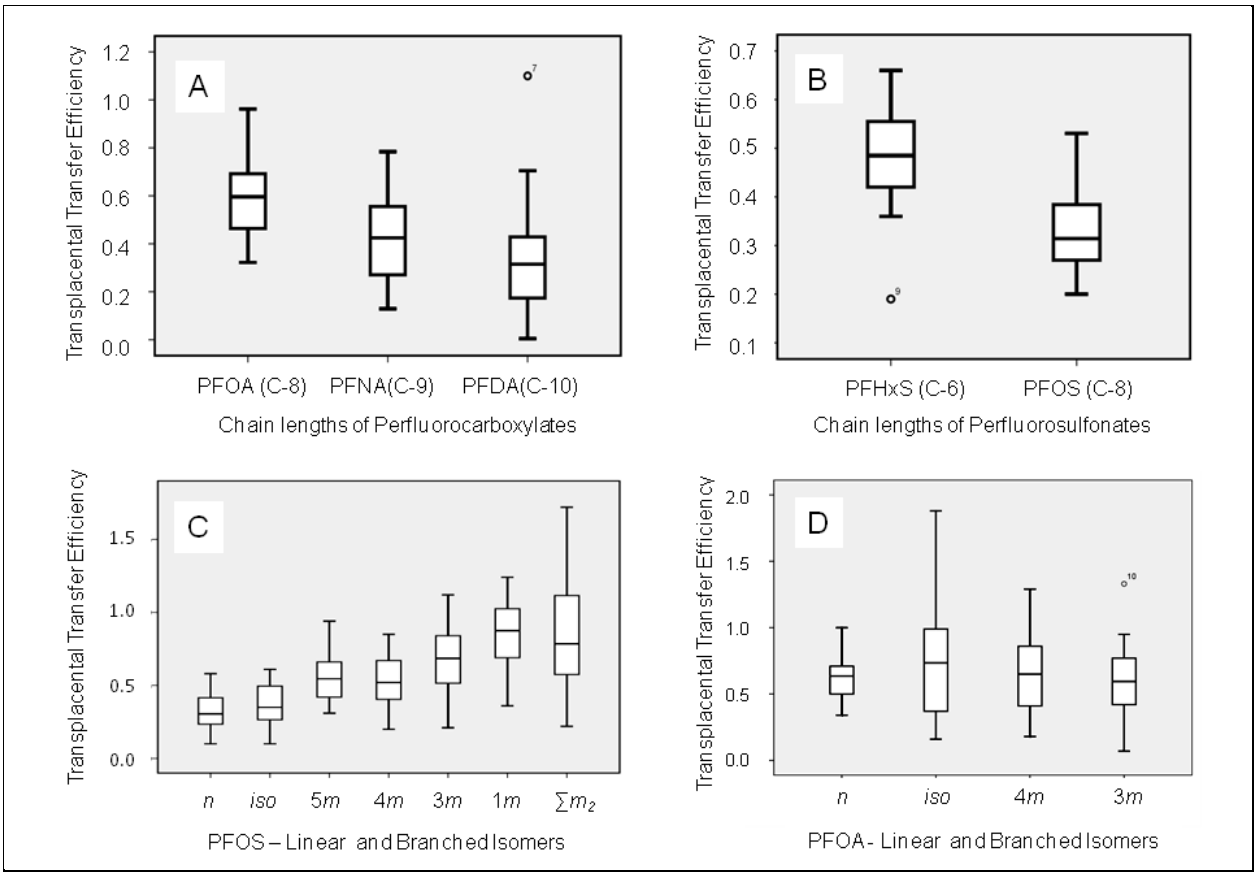

Figure 2.5 TTE distributions for (A) different chain-length perfluorocarboxylates, (B) different chain-length perfluorosulfonates, (C) linear and branched PFOS isomers, and (D) linear and branched PFOA isomers. The upper and lower bounds of the boxes indicate the 75th and 25th percentiles, respectively, and the horizontal lines within the boxes indicate median values. The upper and lower limits of the whiskers indicate minimum and maximum values, respectively, and points above or below the whiskers indicate outlier values.

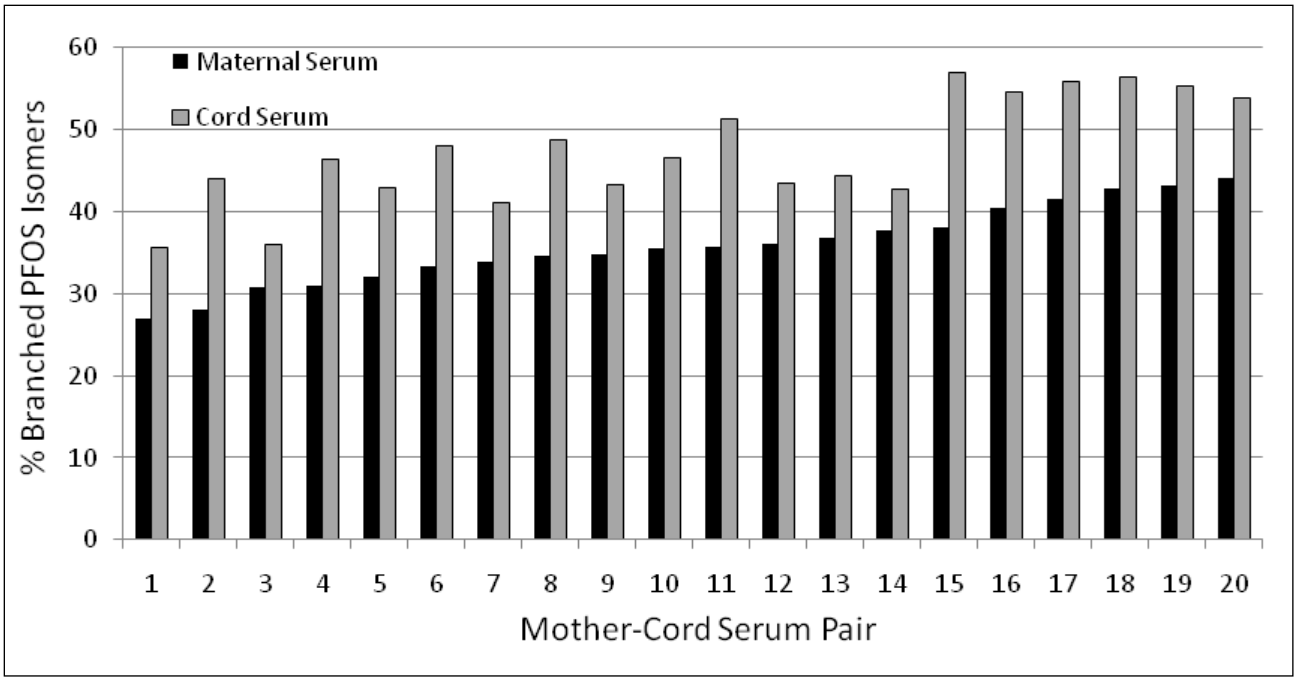

Figure 2.6. Percent branched PFOS isomers (Σbranched/ (Σbranched+linear)) in 20 matched samples of maternal serum (15 weeks gestation) and cord serum at delivery. Samples are arranged, from left to right, by increasing branched PFOS isomer content of the maternal sample.

Unlike in *Hanssen et al. (2010)*, where total branched PFOS isomers were quantified together, we analyzed individual branched isomers, and results suggest a structure-activity relationship for TTE. Specifically, among the perfluoromethyl PFOS branched isomers, TTE increased as the branching point moved closer to the sulfonate moiety: 1*m* > 3*m* > 4*m* ≈ 5*m* > *iso* (Figure 2.4 C). In fact, for 1*m*-, 3*m*-, and particularly $\sum m_2$-PFOS, the concentrations were sometimes higher in cord serum than in corresponding maternal serum (resulting in maximum TTE values > 1.0), which was never the case for total PFOS or linear PFOS (Table 2.5).

Branched PFOA isomers contributed 0.43-4.3 % (mean 1.9 %) of total PFOA in maternal serum, and from 0.71-5.7 % (mean 2.2 %) in cord serum. Such highly linear isomer profiles of PFOA in human serum have previously been reported *(De Silva and Mabury 2006),* yet it is important to note that these cannot be used to quantitatively assess exposure sources (i.e. telomer versus electrochemical) because in animal models the branched isomers of PFOA are accumulated to a lesser extent than linear PFOA *(De Silva et al. 2009).* There was no structure-activity evident for PFOA isomers (Figure 2.4D), but a paired t-test indicated significantly higher total branched PFOA isomers in cord serum than maternal serum (p=0.02). In some cases, the concentrations of 5*m*-, 4*m*-, and 3*m*-PFOA were higher in the cord serum than in corresponding maternal serum (resulting in maximum TTE > 1.0), which was never the case for total PFOA or linear PFOA (Table 2.5).

Table 2.5. Transplacental transfer efficiency (TTE) calculated by the ratio of cord: maternal serum concentrations. Values less than 1 indicate higher concentrations in maternal serum, whereas values greater than 1.0 indicate higher concentrations in cord serum.

	Total, linear and branched PFOS								Total, linear and branched PFOA							Other PFCs		
Value	Total PFOS	n-PFOS	iso PFOS	5m-PFOS	4m-PFOS	3m-PFOS	1m-PFOS	$\sum m_2$-PFOS	Total PFOA	n-PFOA	iso-PFOA	5m -PFOA	4m -PFOA	3m -PFOA	tb-PFOA	Total PFNA	Total PFDA	Total PFHxS
Arithmetic Mean	0.33	0.33	0.36	0.53	0.55	0.67	0.87	0.84	0.61	0.62	0.84	0.86	0.64	0.76	0.25	0.41	0.34	0.41
Median	0.31	0.30	0.34	0.52	0.52	0.68	0.88	0.78	0.63	0.61	0.67	0.54	0.68	0.68	0.25	0.38	0.23	0.38
Standard Deviation	0.09	0.12	0.14	0.18	0.19	0.23	0.23	0.37	0.17	0.20	0.58	0.99	0.34	0.59	0.32	0.17	0.25	0.12
Minimum	0.20	0.10	0.09	0.25	0.20	0.21	0.36	0.22	0.32	0.26	0.16	0.09	0.09	0.07	0.02	0.13	0.00	0.29
Maximum	0.53	0.58	0.60	0.93	0.85	1.12	1.24	1.72	0.96	1.00	2.56	2.26	1.29	2.74	0.48	0.78	1.10	0.56
N [a]	20	20	20	20	20	20	20	20	20	20	20	4	19	18	2	20	16	8

[a] number of maternal-cord pairs that were available for calculating TTE. When concentrations were non-detect in maternal or cord samples, that pair was excluded in the analysis. Mean TTEs were always below 1.0, indicating lower concentrations in the cord serum than maternal serum (all $p<0.01$). Linear PFOS and PFOA are denoted as n-PFOS and n-PFOA respectively.

Simple diffusion is often the mechanism by which chemicals cross the placental barrier *(Syme et al. 2004),* thus the TTE of hydrophilic compounds is generally lower than for hydrophobic compounds *(Van der Aa et al. 1998).* Based on earlier elution in reversed-phase chromatography, branched PFOS isomers are anticipated to be more hydrophilic than linear PFOS, and short-chain carboxylates (e.g. PFOA) should be more hydrophilic than longer-chain carboxylates (e.g. PFNA and PFDA), thus the current results are unexpected. However, perfluorinated acids are highly protein bound in serum *(Jones et al. 2003),* and the dynamics of protein binding are likely to influence TTE. For example, if the binding affinity of linear PFOS to maternal serum protein is higher than for branched PFOS isomers, there would be a higher free fraction of branched PFOS available to cross the placenta.

For the major PFCs in maternal serum we examined whether the branched isomer content was correlated to the branched isomer content in the corresponding house dust sample. While we did not observe a significant correlation between serum and dust branched isomer content for PFOS (Spearman Correlation Coefficient=-0.10, p=0.35) or PFHxS (Spearman Correlation Coefficient=-0.11, p=0.33), we found a borderline significant correlation for PFOA (Spearman Correlation Coefficient=0.35, p=0.08). However, we cannot confirm that dust was a source of branched PFOA isomers in these women given the small sample size (n=20) and the potential contribution of other sources of exposure, including diet, water, and air *(Haug et al. 2011).*

In contrast with expectations, we observed a higher mean percentage of branched PFOS isomers in maternal serum [36 %, 95% confidence interval (CI) 33.6 to 38.2 %] than in historic 3M Co. ECF PFOS (30 % CI 29.3 to 30.7 %) *(Reagen et al. 2007)* or house dust samples (30% CI 28.6 to 31.3 %). A paired t-test showed significantly higher branched PFOS content in maternal serum than in house-dust (6% higher, 95 % CI 3.1 % - 8.9 %, p <0.001). Studies in rodents show that branched PFOS isomers are no more bioaccumulative than linear PFOS *(De Silva et al. 2009)*, thus it would seem pharmacokinetically impossible to accumulate >30 % branched PFOS isomers if the only source of exposure was ECF PFOS. Nonetheless, *Karrman et al. (2007)* and *Hanssen et al. (2010)* also reported high proportions of branched PFOS isomers in human serum. While it is possible that PFOS isomer pharmacokinetics in humans are opposite to those in rats *(De Silva et al. 2009)*, or that some humans are exposed to an unusually high branched PFOS source in the diet, an alternative explanation is that a significant proportion of the PFOS body burden comes from metabolism of PFOS-precursors. *Benskin et al. (2009)* demonstrated that branched isomers of a PFOS-precursor could be biotransformed at greater rates than the corresponding linear precursor and *Haug et al. (2011)* found a significant association between PFOS-precursors in air and increasing branched PFOS content of serum. In the current samples it was evident that maternal and cord serum PFOS concentrations were higher (p<0.01 for maternal serum, p=0.01 for cord serum) when N-methyl perfluorooctanesulfonamidoacetate (a PFOS-precursor) was detected in the same

sample, but no significant association was found between total dust PFOS-precursors and the branched PFOS content of serum (p=0.47).

2.3.5 Study Limitations.

One limitation of this study is the relatively small sample size. Larger studies are recommended to elucidate the relative importance of ECF- and telomer- derived sources of PFCs to humans in other areas. The current study was not designed to test whether PFC signatures in dust were responsible for PFC signatures in maternal or cord serum; rather it was an exploratory investigation of the variability of isomer profiles in dust to elucidate manufacturing sources, and of the variation of isomer profiles between maternal and cord samples in order to investigate if branched isomers crossed the placenta to different extents.

A second limitation was that the time of sampling of pregnant women (15 weeks) was relatively early in the pregnancy, and it is not clear if the isomer profile may have been different at time of delivery. For two women in our study we also analyzed 18 week serum samples, and total PFOS and individual PFOS isomers were not substantially different over these 3 weeks (data not shown). Although this is a narrow window of time, it is not an insignificant period because hematologic indices change significantly beginning as early as the 7^{th} week of pregnancy, including expansion of total blood plasma volume by 16 % between 12 and 20 weeks *(Whittaker et al. 1996).*

2.6 Conclusion

Both ECF and telomer manufacturing sources contributed to household dust PFOA concentrations in this exploratory study. Some homes with the highest PFOA dust concentrations had a near exclusive telomer PFOA signal, and such results may help explain why PFOA continues to be a major contaminant of human serum despite the ECF PFOA phase-out. Larger-scale studies that examine manufacturing sources while simultaneously accounting for dietary pathways would be beneficial. It is recognized that such investigations are technically challenging because isomer profiles in biological samples (i.e. food) may bias source apportionment due to differential uptake of the various isomers. The TTE of PFCs was inversely related to chain-length, and TTEs suggest that most branched PFOA and PFOS isomers crossed the placenta to a greater extent than the corresponding linear isomer. In some cases, minor PFOA and PFOS branched isomers were more concentrated in cord serum than maternal serum, indicating that isomer-specific analysis should be performed in future studies of PFCs and birth outcomes.

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CHAPTER 3: Exceptionally High Serum Concentrations of Perfluorohexane Sulfonate in a Canadian Family Linked to Home Carpet Treatment Applications

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Abstract

Perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) are normally the dominant perfluoroalkyl substances (PFASs) in human serum, but here a Canadian family of seven was identified with particularly high exposure to perfluorohexane sulfonate (PFHxS). Disproportionately high serum PFHxS concentrations (range 27.5 – 423 ng/mL) and moderately high PFOS (range 15.2 – 108 ng/mL) and PFOA (range 2.40 – 9.23 ng/mL) concentrations were detected in the family members, with all three chemicals being highest in the youngest children. We therefore sought to identify the source(s) and pathway(s) of this unusual exposure, and to study the excretion of PFASs for this family. Serum, urine, and stool were sampled from family members, carpet, dust, and air were sampled in the home, and a questionnaire was administered. Over 15 years, the family's household carpets were treated 8 times with Scotchgard™ formulations.

Elevated concentrations of PFHxS were detected in household dust (2780 ng/g dust) and in family room carpet (2880 ng/g carpet), and the primary mode of excretion for the major PFASs was through urine. The high PFHxS and moderately high PFOS concentrations in serum and household samples are consistent with the known PFAS content of certain Scotchgard™ formulations, and exposure was likely through dust ingestion and/or inhalation.

3.1 Introduction

Perfluoroalkyl substances (PFASs) are a group of human-made organofluorine compounds that have been manufactured since the 1950s. Owing to their unique properties, including excellent chemical and thermal stability, PFASs have been used in multiple consumer products and in industrial processes. These include household products such as soil repellents on carpets, stain repellents on fabrics, in waxes, polishes, paints and adhesives *(Kissa 1994; Posner 2012).*

Multiple cross-sectional human biomonitoring studies have been conducted around the world for PFASs. Over the past decade, the National Health And Nutrition Examination Surveys (NHANES) in the U.S. have consistently shown that perfluorooctanesulfonate (PFOS) was the dominant PFAS in human blood, followed by perfluorooctanoate (PFOA) *(Kato et al. 2011).* PFOS is also the predominant PFAS in wildlife *(Giesy and Kannan. 2001),* and in the indoor environment *(Fraser et al. 2012, Shoeib et al. 2011)* Not surprisingly, toxicological investigations of PFASs have focused mainly on PFOS and PFOA.

Most human epidemiology studies of PFASs have also focused on PFOS and PFOA, but two recent studies by *Hoffman et al. (2010)* and *Stein and Savitz (2010)* found statistically significant positive associations (OR = 1.06; *95% CI*: 1.02-1.11 and OR= 1.59; *95% CI*: 1.21–2.08) respectively between serum PFHxS concentrations in children and attention deficit hyperactivity disorder (ADHD). However, neither of these studies controlled for socio-economic status which may be an important confounding variable in the association between serum PFAS levels and medical diagnosis of ADHD. Furthermore, the study by Gump et al. (2011) showed a consistent association between serum PFHxS levels and impulsivity, which is an important feature of ADHD (albeit no clinical assessment of ADHD was made in this study). However, based on Danish National Birth Cohort data, *Fei and Olsen (2011)* reported no statistically significant associations between behavioral and motor coordination problems in seven year old children and maternal background serum levels of PFOS and PFOA during pregnancy. Despite that perfluorohexanesulfonate (PFHxS) is known to have a much longer elimination half-life (arithmetic mean 8.5 years, geometric mean 7.3 years) in humans, compared to PFOS (arithmetic mean 5.4 years, geometric mean 4.8 years) or PFOA (arithmetic mean 3.8 years, geometric mean 3.5 years) (Olsen et al. 2007), few studies have focused on its disposition or toxicity. One study by the 3M Co. *(Butenhoff et al. 2009)* reported no reproductive or developmental toxicity associated with PFHxS exposure in rats, and *Sundstrom et al.(2012)* reported that the serum elimination half-life of PFHxS was four months in monkeys, and one month in rodents. Furthermore, *Bijland et al. (2011)* compared

the effects of PFBS, PFHxS and PFOS on mice and found a chain length dependent decrease of plasma triglycerides and total cholesterol due to impairment of lipoprotein production. Recently, *Cassone et al. (2012)* reported significant changes in genes regulating tissue development, cellular organization, and cell-to-cell signaling in chicken embryos exposed in ovo to PFHxS.

At around the time when PFOS was found to be globally distributed in wildlife *(Giesy and Kannan. 2001)* and humans *(Hansen et al. 2001)* the main manufacturer of PFOS in North America, the 3M Company, voluntarily phased-out its electrochemical fluorination (ECF) production of both PFOS and PFOA (3M Company. 2002). PFOS in American serum then declined from a geometric mean (GM) of 30.4 (*95% CI*: 27.1-33.9) ng/mL in 1999, to 20.7 (*95% CI*: 19.2-22.3) ng/mL in 2003, to 17.1 (*95% CI*: 16.0-18.2) ng/mL in 2005, and to 13.2 (*95% CI*: 12.2-14.2) ng/mL in 2007. However, the temporal trend for serum PFHxS over the last decade has not been consistent. After an initial decline from a geometric mean of 2.13 (*95% CI*: 1.91-2.38) ng/mL in 1999 to 1.93 (*95% CI*: 1.73-2.16) ng/mL in 2003, and down to 1.67 (*95% CI*: 1.42-1.98) ng/mL in 2005, serum PFHxS concentrations in Americans then showed a slight upward trend in 2007, with geometric mean levels of 1.96 (*95% CI*: (1.76-2.17) ng/mL in the general population, and 2.39 (*95% CI*: 2.09-2.75) ng/mL among children 12 to 19 years old.

In the recent Canadian Health Measures Survey (CHMS) (*n*=2880) (Health Canada. 2010), PFOS was the predominant PFAS in serum with a geometric mean of 8.85 ng/mL (CI 7.97-9.82), followed by PFOA (2.52 ng/mL; CI 2.36-

2.69) and PFHxS (2.26 ng/mL; CI 1.97-2.59). However, it is interesting to note that the arithmetic mean (AM) for PFHxS (AM 3.87 ng/mL CI: 3.25-4.48) surpassed PFOA (AM 2.89 CI: 2.72-3.07) as the second most prominent PFAS. A detailed examination of the CHMS data show that at 75th, 90th, and 95th percentiles are GM values for PFOA are lower than PFHxS; 3.62, 4.69, and 5.50 ng/mL for PFOA and 3.74, 7.36, and 12.49 ng/mL for PFHxS, respectively. This trend is reversed at the lower centiles. For example, at the 10th, 25th, and 50th percentiles PFOA levels exceed PFHxS levels; 1.32, 1.90, and 2.66 ng/mL for PFOA and 0.74, 1.27, and 2.22 ng/mL for PFHxS, respectively. These figures suggest a greater tail of distribution of PFHxS compared to PFOA in the Canadian population. Furthermore, the ratio of serum levels in males compared to females (at the geometric mean level) is much higher for PFHxS (2.03) compared to PFOA (1.35) and PFOS (1.57), suggesting that PFHxS may be the main driver for gender specific exposure differences to PFAS in the general Canadian population. This unusual pattern of PFHxS distribution in Canadians has not been observed in other cross-sectional studies in other countries, including in the US NHANES studies.

Here we report on a study of a Canadian family who was identified as having unusually high levels of serum PFHxS, and moderately high levels of PFOS and PFOA. This finding led us to investigate and to characterize the possible source and pathway of exposure to PFASs in this family.

3.2 Materials and Methods

3.2.1 Study design

The present study was initiated after a physician in the Province of Alberta (Dr. S. Genuis) contacted our research group in 2008 to report the unusual case of a husband and wife with abnormally high serum levels of PFHxS. This unexpected finding was made during laboratory testing conducted as part of a clinical research project where the couple was enrolled as volunteer control participants in a study on the elimination of chemical toxicants *(Genuis et al. 2011).* For the current work, ethical clearance was subsequently obtained from the Biomedical Panel of the Health Research Ethics Board (University of Alberta) to administer a questionnaire, to take household samples, and to take biological samples from all members of this family (n=7) to study exposure, sources, and excretion of PFASs in each family member. This high socioeconomic status family consisted of a 52 year old father, a 48 year old mother, four sons (aged 23, 21, 17 and 15), and an 18 year old daughter. All members of the family lived in the same household, except that the second son had moved out 3 years prior to sampling. The father is a scientific researcher and the mother is highly educated. At the time of sampling the 2 eldest sons were university students and the 3 younger children were in high school. According to gender and age, the family members will hereafter be referred to as M-52 (father), F-48 (mother), M-23 (first son), M-21 (second son), F-18 (daughter), M-17 (third son) and M-15 (fourth son). None of the family members had reported any conditions requiring medical follow-up to their family physician.

3.2.2 Home Characteristics

The family's house was built in the Edmonton area in 1989, with the exclusive source of heat coming from a boiler-fed in-floor radiant heating system. In many rooms, wall-to-wall carpeting had been installed on top of the heated floors. The house had a finished basement, a main floor consisting of a kitchen, family room, dining room and study rooms, a second floor with 5 bedrooms, and an attic which served as an office for the father. Historically, the house had no fan-forced air circulation system and there was also no fresh-air intake to the house from the outdoors. At the time of the home sampling, renovations had just begun in the basement to improve indoor air circulation, and to install a fresh-air intake system. A questionnaire based interview was administered to the mother to record potential sources of PFASs, household characteristics, dietary habits for all family members, and occupational information.

3.2.3 Sampling

With informed consent, samples of blood, urine and stool were collected from all family members in November 2008. Blood (10 mL) was collected from each participant in plain Vacutainer® tubes (Becton Dickinson, NJ USA) at a licensed private laboratory (DynaLIFE$_{\mathbf{Dx}}$, Diagnostic Laboratory Services, Edmonton, Alberta) and sent to the University of Alberta within one hour of collection. The clotted blood was centrifuged, serum samples aliquoted in 15 mL Corning® polypropylene tubes and stored at -80°C, pending extraction and analysis. The family was given detailed instructions on how to collect the urine and stool

samples in methanol rinsed polypropylene containers which were subsequently stored at -80°C pending analysis.

Carpet, house dust, and indoor air samples were collected from the house in September 2008, in a narrow window of opportunity between getting to know the family and a major renovation to the house whereby all carpets were removed. Rectangular pieces of carpet (approximately 5 cm × 2 cm) were sampled from the basement, family room, dining room, attic, and from bedrooms of the parents and children currently living in the house using scissors. The carpets in all rooms were identical and had been installed at the same time. We also sampled two additional pieces of the same carpet that had never been installed, or treated by Scotchgard™, but had been stored in the basement for many years (referred to hereafter as "untreated carpet"). Nitrile gloves were worn and changed between samples from different rooms, and scissors were rinsed with HPLC-grade methanol before each sample was cut. The carpet samples were stored in 50 mL polypropylene Corning® tubes at -20°C. On the same day that carpets were sampled, the vacuum bag from the central vacuum system was removed and its contents were emptied into a polyethylene bag which was then stored at -20°C.

Indoor air samples (n=2) were collected in the main floor family room and basement in September 2008 using an active air sampling method (4.3 L/min) using Occupational Safety and Health Administration (OSHA) versatile sampler tubes (XAD-2 OVS (Glass Fiber Filter), 13 to 8 × 75 mm size, SKC Inc**.** PA, USA) (Figure 3.1).

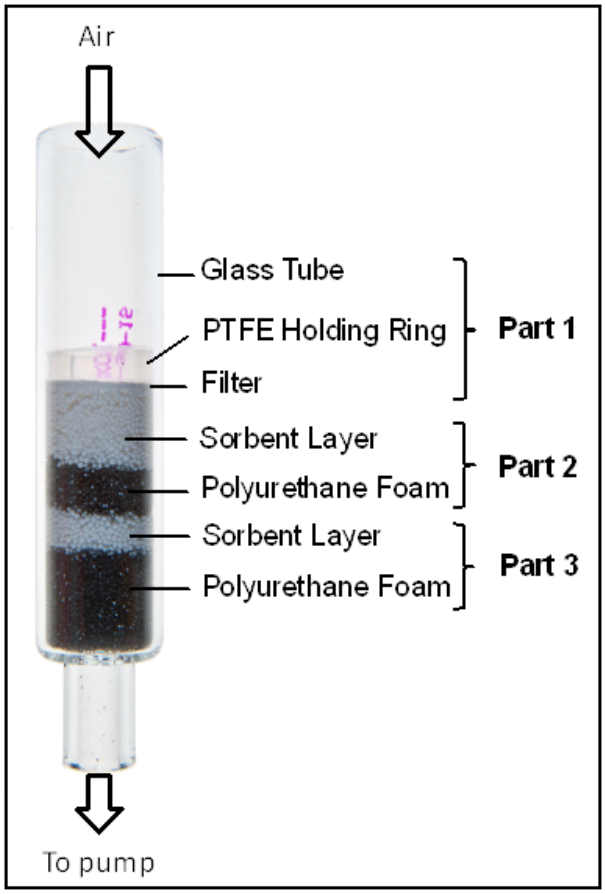


Figure 3.1. OSHA Versatile Sampler (OVS) tube used for air sampling. Parts 1, 2 and 3 were analysed separately.

The pump (Airchek Sampler-Model 224-PCXR8, SKC Inc, PA USA) was calibrated using a Dry Cal DC-Lite primary flow meter (Bios International Corporation, Butler, NJ, USA) using a 5 point calibration protocol. A sample of vacuum dust was again collected in January 2012 to assess any change with respect to 2008. PFAS extraction from the different media was done according to previously published methods, details of which are given in the next section.

3.2.4 Sample preparation

3.2.4.1 Serum

PFASs were extracted from serum using the protein precipitation method of *Flaherty et al. (2006).* Briefly, 20 ng of internal standard (MPFAC-MXA) (Table 3.1) was added to 150 µL of serum, followed by the addition of 150 µL of methanol. The contents of the tube were thoroughly vortex mixed, followed by 15 minutes centrifugation at 10,000 rpm for 15 minutes. Then, 500 µL of 0.1 M formic acid was added to 200 µL of the supernatant, and the mixture was vortexed before analysis. Extraction efficiencies for serum and other matrices are summarized in Table 3.2.

LC-MS/MS				GC/MS			
Native (MPFAC-MXB)		Isotopically Labelled (MPFAC-MXA)		Native		Isotopically Labelled	
Full Name	*Acronym*	*Full Name*	*Acronym*	*Full Name*	*Acronym*	*Full Name*	*Acronym*
Perfluorobutanoic acid	PFBA	Perfluoro [1,2,3,4-$^{13}C_4$] butanoic acid	MPFBA	2-Perfluorohexylethanol	6:2 FTOH		
Perfluoropentanoic acid	PFPeA			2-Perfluorooctylethanol	8:2 FTOH	2-Perfluorooctyl-[1,1-$^{13}H_2$]-[1$^3C_2$]-ethanol	M-8:2 FTOH
Perfluorohexanoic acid	PFHxA	Perfluoro[1,2-$^{13}C_2$] hexanoic acid	MPFHxA	2-Perfluorodecylethanol	10:2 FTOH		
Perfluoroheptanoic acid	PFHpA			Methyperfluoro-1-octane sulfonamidoethanol	N-MeFOSE	2-(N-deuteriomethylperfluoro-1-octanesulfonamido)-1,1,2,2-tetradeuterioethanol	d9-N-MeFOSE-M
Perfluorooctanoic acid	PFOA	Perfluoro[1,2,3,4-$^{13}C_4$] octanoic acid	MPFOA	Ethylperfluoro-1-octane sulfonamidoethanol	NEtFOSE	2-(N-deuterioethylperfluoro-1-octanesulfonamido)-1,1,2,2-tetradeuterioethanol	d9-N-EtFOSE-M
Perfluorononanoic acid	PFNA	Perfluoro[1,2,3,4,5-$^{13}C_5$] nonanoic acid	MPFNA				
Perfluorodecanoic acid	PFDA	Perfluoro[1,2-$^{13}C_2$] decanoic acid	MPFDA				
Perfluoroundecanoic acid	PFUdA	Perfluoro[1,2-$^{13}C_2$] undecanoic acid	MPFUdA				
Perfluorododecanoic acid	PFDoA	Perfluoro[1,2-$^{13}C_2$] dodecanoic acid	MPFDoA				
Perfluorotridecanoic acid	PFTrDA						
Perfluorotetradecanoic acid	PFTeDA						
Perfluorobutane sulfonate	PFBS						
Perfluorohexane sulfonate	PFHxS						
Perfluorooctane sulfonate	PFOS						
Perfluorodecane sulfonate	PFDS						

Table 3.1. Chemical Standards used for LC-MS/MS and GC/MS. All chemical standards used for the current project were purchased from Wellington Laboratories (Guelph, ON, Canada). Specific isomers for PFOS and PFOA (authentic standards of branched PFHxS are **not** available) were characterized based on standards provided by 3M Co., as well as Br-PFOS and T-PFOA standards from Wellington Laboratories.

Table 3.2. Spike and recovery data. Serum, dust and carpet samples were spiked with native standards, while the recovery data presented here for urine and stools are those for isotopically labelled standards. For the air samples spike and recovery was done using native standards on the

	% Recoveries						% Recoveries
Non-Volatile PFASs	*Serum*	*Urine*	*Stools*	*Dust*	*Carpet*	**Volatile PFASs**	*Air (XAD)*
PFOA	96.0	107	88	87.0	112	6:2 FTOH	99.5
PFNA	92.0	94.0	72	89.0	79.0	8:2 FTOH	127
PFDA	112	89.0	64	-	-	10:2 FTOH	88.9
PFUdA	96.0	105	46	-	-	N-MeFOSE	114
PFHxS	95.0	99.0	79	98.0	95.0	N-EtFOSE	91.0
PFOS	95.0	103	83	116	120		

XAD component of the OVS tube

3.2.4.2 Urine

A portion of each urine sample (10 mL) was measured into a 50 mL polypropylene tube, and 15 mL of 1% formic acid in water was added to each tube along with 5 ng of internal standard (MPFAC-MXA). The samples were vortexed for 2 minutes and sonicated for 30 minutes. Each sample was slowly passed through an Oasis HLB (60 mg, 6 mL) SPE cartridge. The SPE cartridge was then eluted with 1 mL of 1% ammonium hydroxide in acetonitrile. The resulting eluant was concentrated to 100 µL and diluted with 200 µL of 90% 0.1 M acetic acid in water/ 10% methanol before analysis.

3.2.4.3 Stool

The method of *Yoo et al. (2009)* for sludge was adapted. Approximately 2 g of each sample was weighed into 15 mL polypropylene tubes, spiked with 10 ng of internal standard (MPFAC-MXA) to which 4 mL aliquots of methanol were added. The mixture was vortexed for 5 min, sonicated for 1 hr., and centrifuged at 3000g for 10 min. A 2 mL portion of the methanol was slowly concentrated down to 100 µL in a premarked vial, and to this 200 µL of 90% 0.1M acetic acid in water/ 10% methanol was added.

3.2.4.4 Dust

Vacuum dust samples were sieved using an AS200 Analytical Retsch sieve shaker and a 150 µm mesh size stainless sieve (Retsch GmbH, Rheinische, Germany). The sieved dust was collected in a methanol rinsed stainless steel pan. The method of *Kubwabo et al. (2005)* was customized for the analysis of the dust

samples. Approximately 0.5 g of sieved dust was weighed into a 15 mL polypropylene tube, spiked with 3.5 ng of each internal standard (MPFAC-MXA) and 5 mL methanol was subsequently added followed by vortexing, sonication and centrifugation. The methanol was decanted and fresh methanol was added 2 more times and the triplicate extracts were pooled. A 5 mL aliquot was transferred to a clean 15 mL centrifuge tube, reduced to 300 μL under nitrogen, and centrifuged for 10 min at 4000g before analysis.

3.2.4.5 Carpet

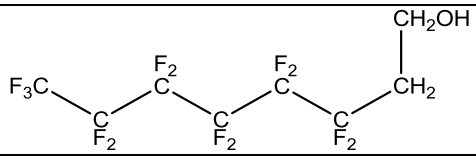
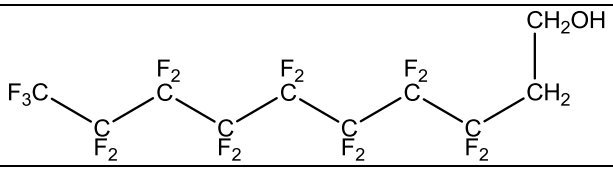
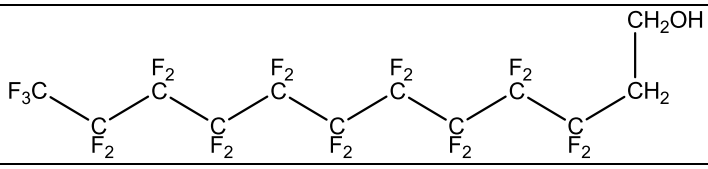
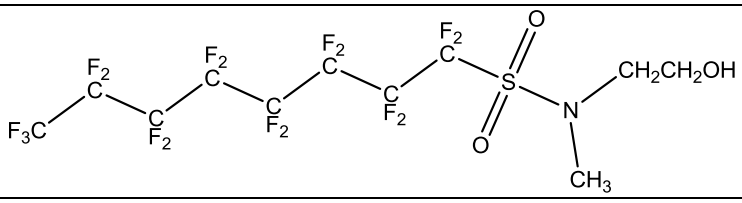
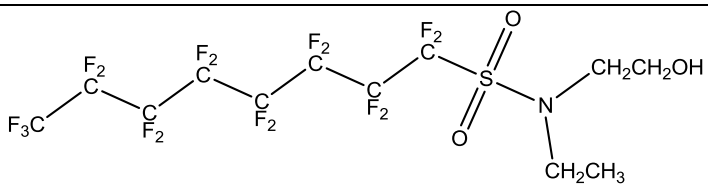
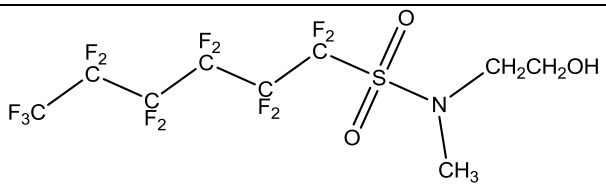
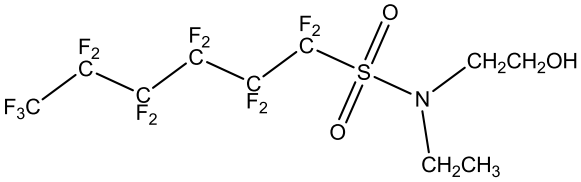
PFAS were extracted from the carpet samples using the method of *L'Empereur et al. (2008).* Briefly, approximately 1 g of each carpet sample was weighed into a 50 mL polypropylene tube, which was then spiked with 3.5 ng of internal standard (MPFAC-MXA). After 1 hour, during which time the internal standard solvent had dried, 10 mL of methanol was added to the 50 mL tube and the tube was vortex mixed for 5 min. The methanol extract was then decanted into a 50 mL polypropylene tube, and the extraction was repeated three more times with 10 mL of methanol. The combined extract was homogenised by vortex mixing and a 2 mL aliquot was concentrated down to less than 100 μL, and resuspended in 200 μL of methanol.

3.2.4.6 Air

Sampling media in the OSHA versatile air sampler (OVS) tubes were separated into 3 fractions for analysis. Fraction 1 contained airborne particles, Fraction 2 contained gaseous airborne analytes, and Fraction 3 was used to examine for

analyte breakthrough (Figure 3.1). Each fraction was spiked with 10 ng of each internal standard: 2-perfluorooctyl-[1,1-$^{2}H_2$]-[$^{13}C_2$]-ethanol (M-8:2 FTOH), 2-(N-deuterioethylperfluoro-1-octanesulfonamido)-1,1,2,2-tetradeuterioethanol (d9-N-EtFOSE-M), 2-(N-deuteriomethylperfluoro-1-octanesulfonamido)-1,1,2,2-tetradeuterioethanol (d9-N-MeFOSE-M) and PFAC-MXA (Table 3.1). After allowing the solvent to dry, this was followed by a double extraction with two aliquots of 1 mL of ethyl acetate. Approximately 800 µL of each extract was then concentrated to 300 µL by a gentle stream of nitrogen in a TurboVap®LV concentration workstation (Caliper Lifesciences, MA, USA) for analysis of N-MeFOSE, N-EtFOSE and possible PFHxS precursors by GC-MS. The remaining unconcentrated extract from each fraction was tested directly for 6:2, 8:2 and 10:2 FTOHs, without prior pre-concentration, as it was found during the method development phase that evaporation lead to substantial loss of these analytes. The same GC-MS conditions used by *Martin et al. (2002)* were used to detect the airborne analytes (Table 3.3). After GC-MS analysis, the extracts of Fraction 1 were evaporated to dryness and the residue reconstituted in 300 µL of methanol for HPLC-MS/MS analysis presence of perfluorinated acids.

Table 3.3. Semi-volatile perfluoroalkyl substances monitored in air samples**.** All the ions monitored were by positive chemical ionization.

Compound	Structural Formula	Acronym	Ions (m/z)
2-Perfluoro hexylethanol		6:2 FTOH	365 327 393
2-Perfluoro octylethanol		8:2 FTOH	465 427 493
2-Perfluoro decylethanol		10:2 FTOH	565 527 593
N-methyl perfluorooctane sulfonamidoethanol		N-MeFOSE	558 540
N-Ethyl perfluorooctane sulfonamidoethanol		N-EtFOSE	572 554 540
N-methyl * perfluorohexane sulfonamidoethanol		N-MeFHxSE	458 440
N-Ethyl * perfluorohexane sulfonamidoethanol		N-EtFHxSE	472 454 440

* denotes the two C-6 compounds which can theoretically be precursors of PFHxS, by analogy to N-EtFOSE and N-MeFOSE.

3.2.5 Total PFAS analysis

Total PFASs (i.e. no isomer separation) were quantified by LC/MS/MS utilizing an Agilent 1100 HPLC coupled to an API 3000 mass spectrometer (Applied Biosystems, Carlsbad, CA, USA) using electrospray ionization in negative ion mode and multiple reaction monitoring (MRM). Chromatographic separation used gradient elution at 600 µL/min on a C-18 150 mm Synergi Hydro-RP column (Phenomenex, Torrance, CA, USA) and a mobile phase consisting of 20 mM ammonium acetate at pH 4 (A) and HPLC grade methanol (B). The interference-free transition (*m/z* 399/119) for PFHxS *(Chan et al. 2009)* was always used for quantification.

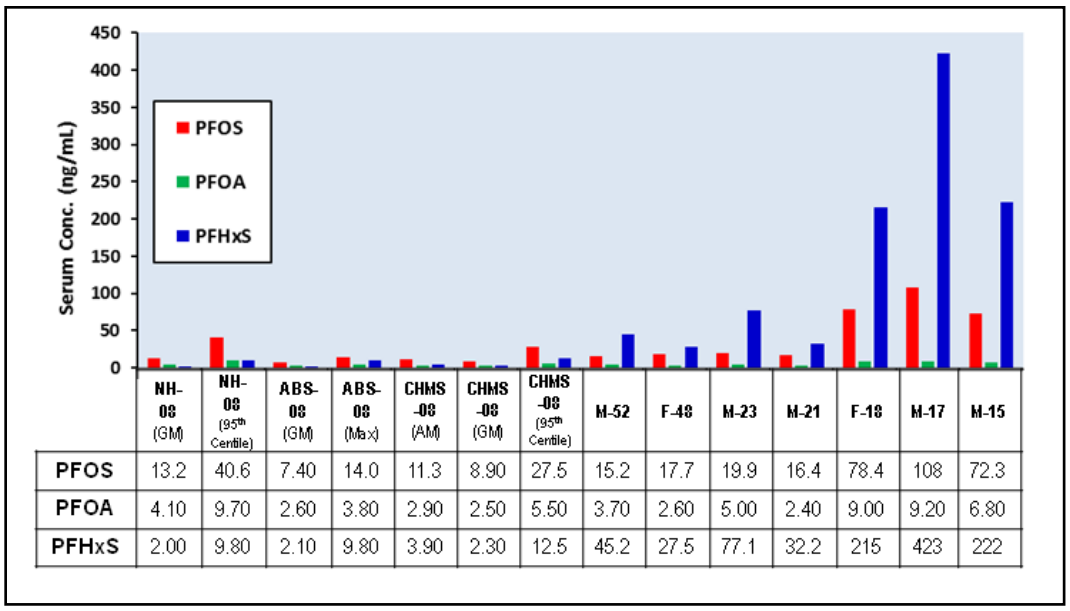
3.2.6 Isomer specific PFAS analysis

The method of *Benskin et al. (2007)* was adapted for isomer specific PFAS analysis of all samples of dust, carpet, and Fraction 1 of the air samples. Chromatographic separation was performed on a FluoroSep RP Octyl column (3µ 100Å, 15 cm × 2.1 mm, ES Industries, West Berlin, NJ) at 150 µL/min. A gradient elution method was used with the mobile phases consisting of A: 5 mM ammonium formate at pH 4.0 and B: methanol. Mass spectral data were collected on a triple-quadrupole mass spectrometer (API 5000, AB Sciex, Concord, ON, Canada) using electrospray ionization operated in negative ion mode.

3.3 Results and Discussion

3.3.1 Serum PFAS concentrations

Serum PFHxS, PFOS and PFOA concentrations among all family members ranged from 27.5 to 423 ng/mL, 15.2 to 108 ng/mL and 2.40 to 9.23 ng/mL, respectively (Figure 3.2).



	NH-08 (GM)	NH-08 (95th Centile)	ABS-08 (GM)	ABS-08 (Max)	CHMS-08 (AM)	CHMS-08 (GM)	CHMS-08 (95th Centile)	M-52	F-48	M-23	M-21	F-18	M-17	M-15
PFOS	13.2	40.6	7.40	14.0	11.3	8.90	27.5	15.2	17.7	19.9	16.4	78.4	108	72.3
PFOA	4.10	9.70	2.60	3.80	2.90	2.50	5.50	3.70	2.60	5.00	2.40	9.00	9.20	6.80
PFHxS	2.00	9.80	2.10	9.80	3.90	2.30	12.5	45.2	27.5	77.1	32.2	215	423	222

Figure 3.2. Serum concentrations of PFOS, PFOA and PFHxS in the current family compared to concentrations in other biomonitoring studies in the United States and Canada. NH-08 – NHANES study 2008, ABS-08 – Alberta Biomonitoring Study 2008, CHMS-08 – Canadian Health Measures Study 2008.

These concentrations are all high and can be compared to the 95th percentiles of the general Canadian population: 12.5 ng/mL PFHxS (15.9 ng/mL for males, 8.52 ng/mL for females), 27.5 ng/mL PFOS (31.3 ng/mL for males, 20.0 ng/mL for females) and at 5.50 ng/mL PFOA (5.98 ng/mL for males and 4.99 ng/mL for females). The age and gender stratified distributions for these three PFASs in the Canadian population are provided in the Figure 3.3.

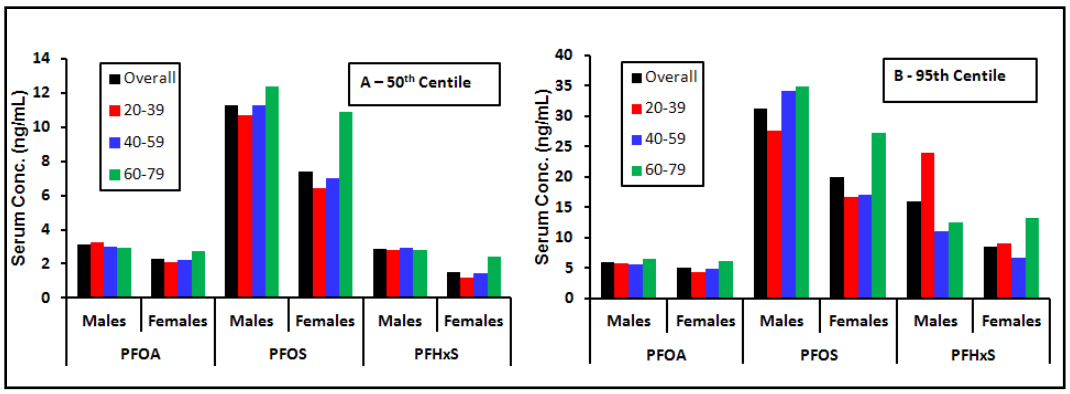


Figure 3.3. Distribution of serum PFOA, PFOS and PFHxS in the general Canadian population, stratified by 3 age categories and gender. Note that at the 95th centile, serum PFHxS in young males is much higher than older males and all females categories. This figure is generated using publicly available data from the Canadian Health Measures Survey. (http://www.hc-sc.gc.ca/ewh-semt/pubs/contaminants/chms-ecms/section8-eng.php#n8_5. Accessed on June 25 2012)

Moreover, in every family member, the relative profile was PFHxS > PFOS > PFOA, which is in stark contrast to what is conventionally observed in human biomonitoring studies *(Kato et al. 2011, Kannan et al. 2004)*, where the general pattern is always PFOS > PFOA > PFHxS. The other PFASs detected in serum were perfluoroheptane sulfonate (PFHpS), N-methyl perfluorooctanesulfonamidoacetate (N-MeFOSAA), perfluorononanoate (PFNA), and perfluorobutanoate (PFBA) (Table 3.4).

Among retired fluorochemical production workers, serum PFHxS has been reported to be in the range of 16-1,295 ng/mL (mean 290 ng/mL), *(Olsen et al. 2007)* but such high serum PFHxS in non-occupationally exposed humans, coupled with high PFOS, has only been reported once previously (up to 718 ng/mL of PFHxS and 515 ng/mL of PFOS) in American children *(Olsen et al. 2004).* Notably, in the present study the concentrations were also highest in the

youngest children. *Kato et al. (2011)* showed that age was inversely related to serum PFHxS levels among Americans, especially at the 95th percentile, and that this consistent trend was evident in all four NHANES studies from 1999 to 2008. This age associated trend in serum levels of PFHxS is also shown in 2 independent Canadian biomonitoring studies on pregnant women *(Govt. of Alberta. 2008)* and children under 13 years *(Govt. of Alberta. 2010)*, whereby serum PFHxS concentrations in children were higher than in all age groups of pregnant women. *Kato et al. (2011)* also showed that males generally have higher serum PFAS than females, in particular for PFHxS, and in the current study the 2 youngest sons (M-17 and M-15) had higher serum PFHxS than the daughter (F-18) (Figure 3.2). The Canadian Health Measures Survey (Figure 3.3) also showed, at the 95th percentile, that serum PFHxS among males was higher than females, and that PFHxS was higher in the youngest age group; albeit the youngest age group was 20 – 39 years, compared to NHANES where 12 – 19 year olds were tested.

3.3.2 PFAS in stool and urine

None of the PFASs were detected in the stool samples, except for perfluorotridecanoic acid (PFTrDA) which was detected in the stool of the second son (M-21), third son (M-17) and the youngest son (M-15), and perfluorotetradecanoic acid (PFTeDA) which was detected in the stool of the second son (M-21). However, PFHxS was detected in the urine of all 5 children but not for the parents. We can only speculate that that this may be due to the fact that the parents have among the lowest serum levels, and so the amount of PFHxS

excreted in their urine is not above the current detection limit of 0.05 ng/mL. PFOS was detected in the urine of the father, the daughter (F-18), and the second son (M-17) (Table 3.4).

Table 3.4. Serum, urine and feces concentrations of different PFASs in family members.

		PFHxS	PFOS	PFOA	PFNA	PFTrDA	PFTeDA	PFHpA	PFHpS	PFBA	N-MeFOSAA
Serum (ng/mL)	**M-52**	45.2	15.2	3.68	-	-	-	-	-	-	-
	F-48	27.5	17.7	2.55	-	-	-	0.63	-	-	1.00
	M-23	77.1	19.9	4.96	0.95	-	-		0.72	0.610	-
	M-21	32.2	16.4	2.40	0.52	-	-	1.92	-	-	-
	F-18	215	78.4	9.02	0.73	-	-	-	2.36	-	0.660
	M-17	423	108	9.23	-	-	-	-	3.01	-	0.870
	M-15	222	72.3	6.84	0.68	-	-	-	1.41	-	0.960
Urine (ng/mL)	**M-52**	-	0.175	-	-	-	-	-	-	-	-
	F-48	-	-	-	-	-	-	-	-	-	-
	M-23	0.052	-	-	-	-	-	-	-	-	-
	M-21	0.054	-	-	-	-	-	-	-	-	-
	F-18	0.324	0.390	-	-	-	-	-	-	-	-
	M-17	0.318	0.074	-	-	-	-	-	-	-	-
	M-15	0.248	-	-	-	-	-	-	-	-	-
Stool (ng/g)	**M-52**	-	-	-	-	-	-	-	-	-	-
	F-48	-	-	-	-	-	-	-	-	-	-
	M-23	-	-	-	-	-	-	-	-	-	-
	M-21	-	-	-	-	4.80	-	-	-	-	-
	F-18	-	-	-	-		-	-	-	-	-
	M-17	-	-	-	-	7.80	3.20	-	-	-	-
	M-15	-	-	-	-	9.10	-	-	-	-	-

The lower limits of detection (LLOD) of the different PFAS are as follows: 0.05 ng/mL for serum and urine and 2.50 ng/g for stools. "- " indicates non-detectable at current LLODs. In addition to the compounds shown in the table, these additional PFAS were analysed but were not detected in any of the three biological media: perfluorobutane sulfonate (PFBS), perfluorodecane sulfonate (PFDS), perfluoro pentanoate (PFPeA), perfluorohexanoate (PFHxA), perfluorodecanoate (PFDA), perfluoroundecanoate PFUdA, perfluorododecanoate (PFDoA), FOSA and N-EtFOSAA.

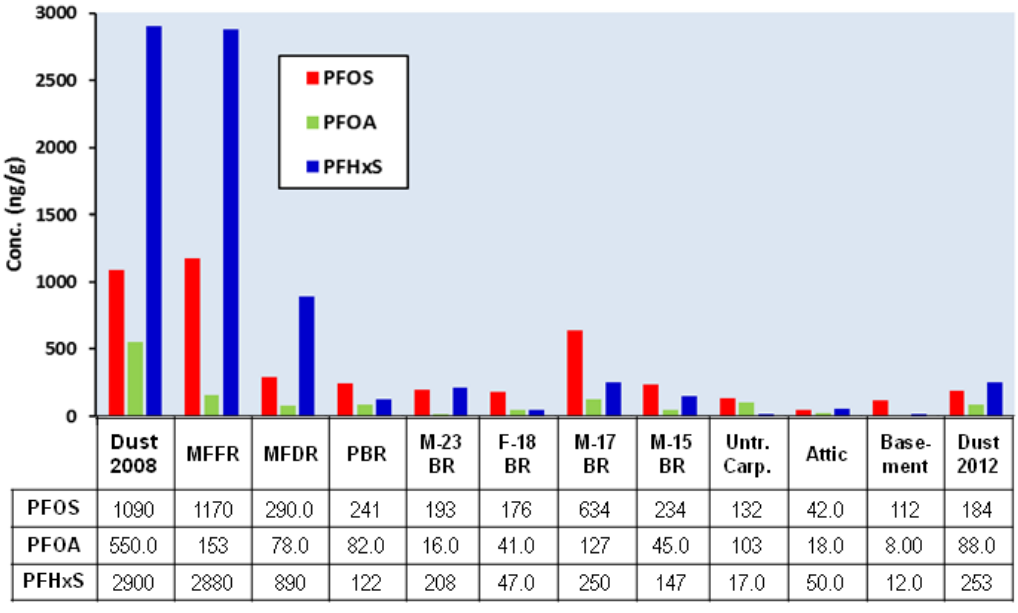
Assuming a daily urinary volume of 1500 mL, urinary PFHxS excretion of the five children ranged from 78.0 - 486 ng/day. Although PFHxS was undetected in the stool samples, at the current detection limit of 2.50 ng/g, based on 100 g of average daily stool excretion in Western countries *(Cummings et al. 1992)* and 75 % moisture content *(Jensen et al.1976),* the maximum daily amount of PFHxS that could possibly have been excreted in stool was 62.5 ng. Thus, it is likely that urine is the primary mode of excretion for PFHxS. To our knowledge this is the first human data on the excretion of PFHxS in urine. Elimination of other PFASs in human urine has been documented by *Harada et al. (2005)* on the renal clearance of PFOA and PFOS, and *Ubel et al. (1980)* on PFOA excretion in urine of fluorochemical plant workers exposed to PFAS, but the current study is the first time that parallel samples of stool have been analyzed.

3.3.3 Vacuum dust and carpet samples

House dust and carpet PFAS concentrations are reported in Figure 3.4. What was surprising, just like in the family's serum, was that PFHxS was the dominant PFAS in the vacuum dust (2780 ng/g PFHxS, compared to 1090 ng/g PFOS) and carpet samples (n=10). The highest concentrations among carpet samples were in the main floor family and dining rooms (Figure 3.4). Such high PFHxS levels in house dust are not unprecedented; for example maximum PFHxS in dust was 4305 ng/g (median: 23.1 ng/g) in Canadian homes *(Kubwabo et al. 2005),* 43765 ng/g (median:185.5 ng/g) in American homes *(Kato et al. 2009),* 6100 ng/g in U.K homes (median:210 ng/g) and up to 34,000 ng/g (median: 700 ng/g) in U.K. classrooms *(Goosey et al. 2011).* Thus, although this family's exposure to PFHxS

in dust is high, many other families and their children may have similar exposure to PFHxS in their homes, or in micro-environments.

Compelling evidence that the major PFASs in vacuum dust originated from the carpets is shown by similar proportions of PFHxS, PFOS, and PFOA between the dust and the carpets with the highest concentrations of PFASs (Figure 3.4), and also by nearly identical isomer profiles (Figure 3.5); for example whereby the total percentage of branched PFHxS (based on peak area in the m/z 399/119 transition) in the 2008 dust and carpet were 33.1% and 33.2% (average of 5 carpet samples), respectively.



	Dust 2008	MFFR	MFDR	PBR	M-23 BR	F-18 BR	M-17 BR	M-15 BR	Untr. Carp.	Attic	Base-ment	Dust 2012
PFOS	1090	1170	290.0	241	193	176	634	234	132	42.0	112	184
PFOA	550.0	153	78.0	82.0	16.0	41.0	127	45.0	103	18.0	8.00	88.0
PFHxS	2900	2880	890	122	208	47.0	250	147	17.0	50.0	12.0	253

Figure 3.4. Distribution of PFOS, PFOA and PFHxS in dust samples (2008 and 2012), and carpets from main floor family room (MFFR), main floor dining room (MFDR), parents' bedroom (PBR), first son's bedroom (M-23 BR), daughter's bedroom (F-18 BR), third son's bedroom (M-17 BR), fourth son's bedroom (M-15 BR), untreated carpet, attic and basement.

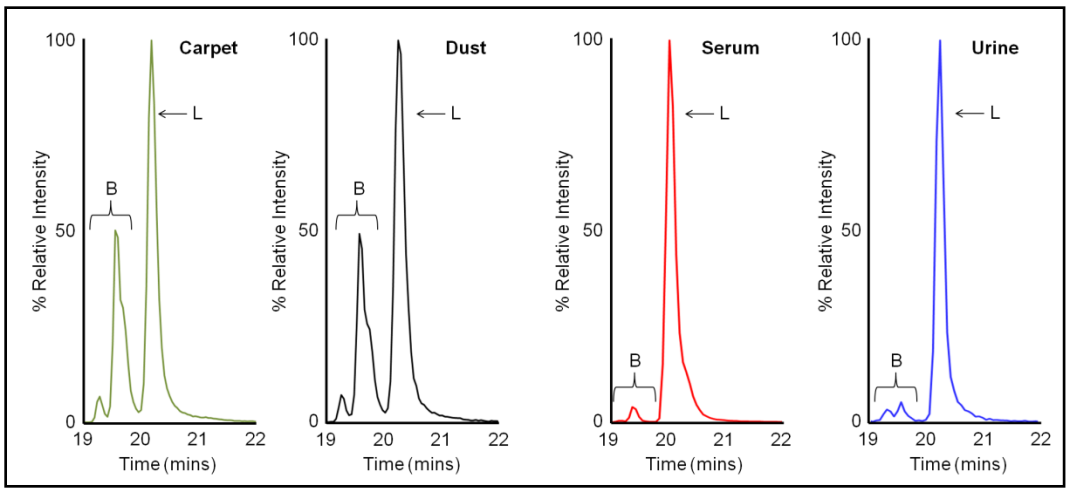


Figure 3.5. Chromatograms showing branched (B) and linear (L) isomers of PFHxS in carpet, dust, serum and urine samples. Note the close similarity between chromatograms of the carpet and the dust sample. The urine sample is slightly enriched in the branched isomer content compared to the serum sample.

Furthermore, the dramatic decline of all three major PFASs in the 2012 vacuum dust (PFHxS: 253 ng/g, PFOS: 184 ng/g, PFOA: 87.6 ng/g) with respect to the 2008 dust (PFHxS: 2780 ng/g, PFOS: 1090 ng/g, PFOA: 516 ng/g) coincided with, and is likely attributable to the removal of all carpets from the house shortly after the 2008 sampling time. Overall, this suggestion is consistent with the findings of *Kubwabo et al. (2005)*, who reported a statistically significant positive correlation (p = 0.0013) between house dust PFAS concentrations and total carpeted area in Canadian houses.

3.3.4 Air samples

Analysis for semi-volatile PFASs (PFOS- and PFOA-precursors) were made only in Fraction 2 of the two air samples taken in 2008 (basement, main floor dining room, respectively). These showed the presence of 2-perfluorooctylethanol (8:2 FTOH) (4640 pg/m^3, 3280 pg/m^3), 2-perfluorodecylethanol (10:2 FTOH) (1470

pg/m^3, 831 pg/m^3), methyperfluoro-1-octane sulfonamidoethanol (N-MeFOSE) (8400 pg/m^3, 1210 pg/m^3) and ethyperfluoro-1-octane sulfonamidoethanol (N-EtFOSE) (1110 pg/m^3, 680 pg/m^3). These indoor air levels are within the range reported for other Canadian houses *(Shoeib et al. 2011).* Although 2-perfluorohexylethanol (6:2 FTOH) was detected in Fraction 2, it was also present in the breakthrough fraction and thus could not be quantified. Possible PFHxS precursors were examined by monitoring for analogous ions to N-EtFOSE and N-MeFOSE, (Table 3.3), but these were not detected.

Subsequent LC-MS/MS analysis of Fraction 1 (particulate filter) from the main floor family room air sample showed detectable levels of PFHxS (27.4 pg/m^3), PFOS (41.6 pg/m^3) and PFOA (19.7 pg/m^3). The collected mass of dust could not be weighed, but based on indoor dust levels of a typical Canadian house (15.2 µg/m^3 air) *(Pellizzari et al. 1999),* the corresponding family room airborne concentrations (by mass of dust) for PFHxS, PFOS and PFOA were estimated to be 1800, 2740 and 1300 ng/g, respectively. These numbers can be compared to the vacuum dust concentration, and are within the same range. Analysis of Fraction1 from the basement air sample showed one order of magnitude higher concentrations of PFOS (393 pg/m^3), PFHxS (426 pg/m^3), and PFOA (453 pg/m^3) compared to the main floor family room air sample. The reason for the very high concentrations is not clear.

3.3.5 Isomer specific analysis

The PFHxS isomer patterns in carpet, dust, serum and urine were compared (Figure 3.5). As previously noted, the isomer pattern of PFHxS in dust was the same as that of the family room carpet, but major differences were observed in all serum samples, whereby the relative amount of branched isomers (based on *m/z* 399/119 peak area) was substantially lower in serum.

Assuming that the carpets are the source of PFASs, and that house dust is the main pathway of PFHxS exposure (by ingestion or inhalation), this finding suggests either preferential absorption of linear PFHxS, or preferential excretion of branched PFHxS isomers. We could not test the first possibility, however based on a qualitative comparison of the PFHxS chromatograms of urine and serum (Figure 3.5), an enriched branched PFHxS isomer signature was seen in urine, consistent with preferential excretion of branched isomers. This is consistent with the findings of *Benskin et al. (2009)*, whereby rats excreted branched PFHxS in urine to a greater extent than linear PFHxS. The same trend was observed by *Benskin et al.(2009)* for PFOA isomers in rats, which is a likely explanation for the much lower branched PFOA content in serum of all family members compared to the dust and carpet sampled in the house (Table 3.5).

Table 3.5. Branched PFOA content in house dust, carpet, air particles, serum samples and a historical 3M ECF reference standard.

Sample	% Branched PFOA
Vacuum Dust	
2008 dust	20.5
2012 dust	15.4
Air Samples (Fraction 1)	
Main Floor Family Room	20.6
Basement	21.8
Carpets	
Main floor family room	19.9
Parents bedroom	21.1
M-23 bedroom	19.4
F-18 bedroom	21.4
M-17 bedroom	21.8
M-15 bedroom	19.3
Untreated	20.7
Serum	
M-52	3.68
F-48	2.09
M-23	1.83
M-21	2.69
F-18	3.51
M-17	4.91
M-15	2.86
Reference Standard	
3M Co. ECF PFOA standard	22.2

PFOA isomer patterns in dust, carpet and air can be used to determine if the source of PFOA is from historical 3M Co. ECF manufacturing or from contemporary telomer manufacturing by other companies, as previously described *(Beesoon et al. 2011).* The reference 3M Co. ECF PFOA standard had a branched content of 22.2% by the current methodology, while all samples of air, carpet, and dust collected in 2008 had a branched content between 19.3% and 21.8%, consistent with the primary source of PFOA in the house being from a historical ECF source (Table 5). Also noteworthy is that by 2012, long after the removal of carpets from the house, PFOA concentrations in house vacuum dust had declined (Figure 3.4), and the source of PFOA was increasingly from a contemporary telomer (i.e. purely linear) manufacturing source of PFOA (15.5% branched PFOA in 2012 dust).

3.3.6 Family and house characteristics

Through the questionnaire based interview, it was determined that the family moved into this new house in 1989, at which time the first 2 sons were already born (5 and 3 years old at that time). None of the family members had any occupational exposure to PFASs. The family rarely ate fast food, or ate in restaurants, and their dietary intake was comparable to an average Canadian, thus excluding the likelihood that food was a major source of exposure. The interview also revealed that the family had carpeting installed in their house in 1989, and in many places this was fixed on top of in-floor radiant heating. Furthermore, since 1991, the family had repeatedly hired the service of the same cleaning company to clean the carpets and to apply Scotchgard™ to the main floor family room, and

occasionally to the dining room. Detailed receipts documenting the specific dates, the rooms where the formulations were applied, and noting the Scotchgard™ trademark (but not the specific formulation) were available. The same company had been hired over the 18 years, with the first application made in 1991, and the last application in 2007, with 6 intermittent applications (Figure 3.6).

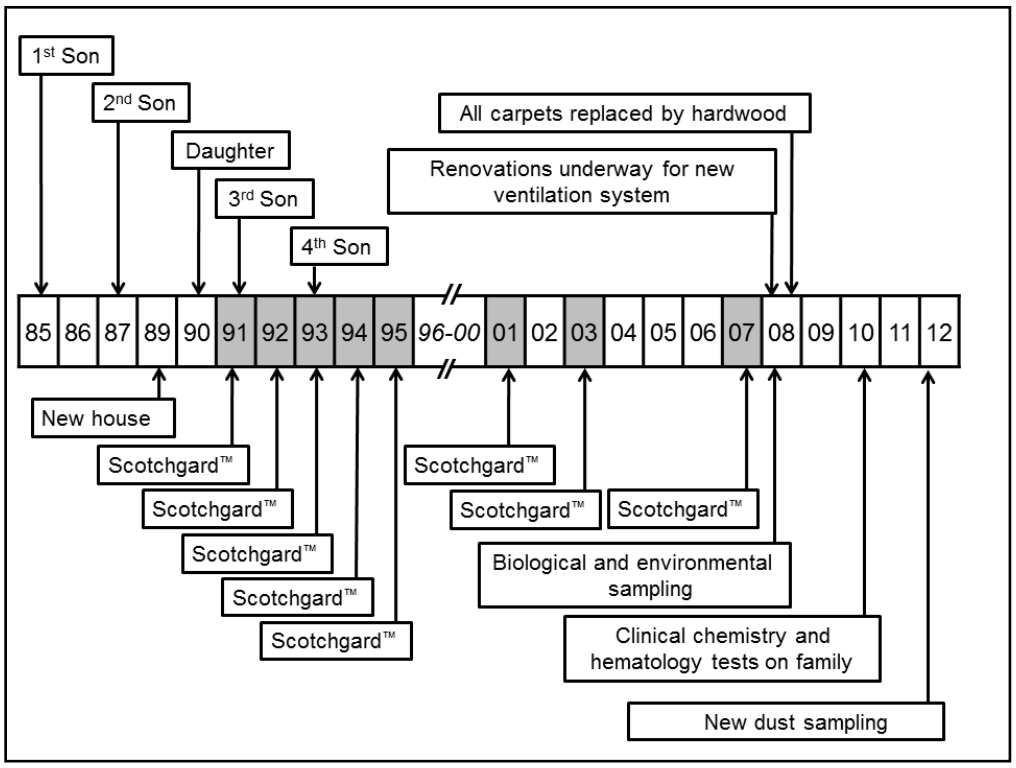
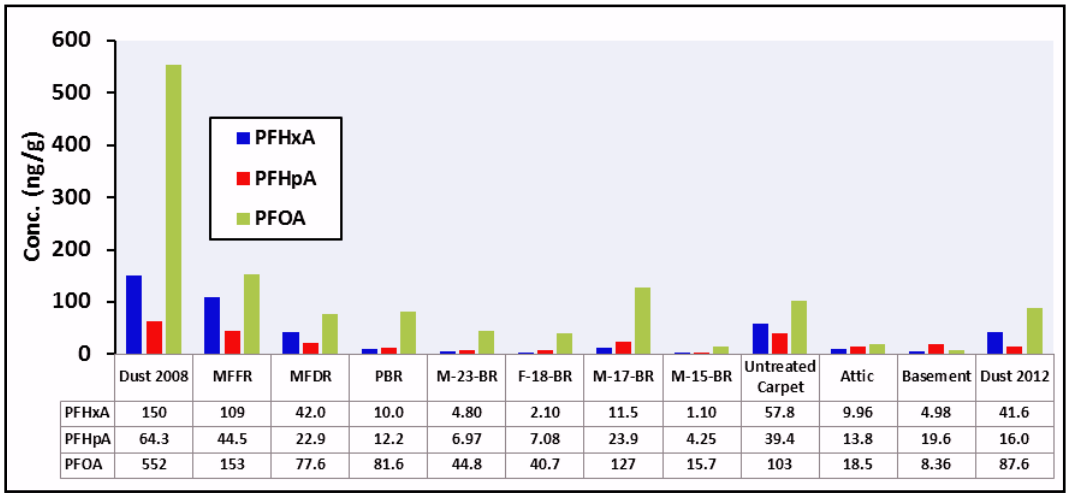


Figure 3.6. Timeline showing Scotchgard™ applications to the main floor family room carpet and occasionally (1995, 2003 and 2007) to the dining room carpet in relation to year in which the children in the family were born and sampling conducted for the current study.

3.3.7 General Interpretation and Implications

Results from PFAS analysis of carpets corroborated the receipts issued by the cleaning company, because the highest concentrations were detected in the family room and dining room carpets where Scotchgard™ was noted to have been applied (Figure 4). The relatively low levels of PFHxS (16.7 ng/g) and PFOS (132 ng/g) in the untreated/unused carpet", is strong evidence that the main source of PFASs in carpet(s) was post-installation treatment with Scotchgard™. PFOA was the major perfluoroalkylcarboxylate in carpets (range 8.36-153 ng/g), including on the untreated carpet (103 ng/g, 20.7% branched), suggestive of a pre-installation ECF source of PFOA (Figure 3.7).



	Dust 2008	MFFR	MFDR	PBR	M-23-BR	F-18-BR	M-17-BR	M-15-BR	Untreated Carpet	Attic	Basement	Dust 2012
PFHxA	150	109	42.0	10.0	4.80	2.10	11.5	1.10	57.8	9.96	4.98	41.6
PFHpA	64.3	44.5	22.9	12.2	6.97	7.08	23.9	4.25	39.4	13.8	19.6	16.0
PFOA	552	153	77.6	81.6	44.8	40.7	127	15.7	103	18.5	8.36	87.6

Figure 3.7. Distribution of PFHxA, PFHpA, and PFOA in dust samples (2008 and 2012), main floor family room (MFFR), main floor dining room (MFDR), parents' bedroom (PBR), first son's bedroom (M-23-BR), daughter's bedroom (F-18-BR), third son's bedroom (M-17-BR), fourth son's bedroom (M-15-BR), untreated carpet, attic and basement.

For the perfluoroalkylsulfonates detected in carpet, the very high levels of PFHxS, relative to PFOS, was unanticipated because it is conventionally believed that the major component of historical 3M Scotchgard™ formulations were C-8 compounds, particularly PFOS and its precursors. However, based on 3M Co. literature submitted to the U.S. EPA *(3M Company. 2000),* PFHxS concentrations in formulations of "SG Carpet Protector Product" and "SG Carpet Protector Treated Web" were 1900 parts per million (ppm), whereas PFOS concentrations were only 610 ppm and 470 ppm in the two products, respectively. Thus, the PFHxS:PFOS ratios in these 2 formulations ranged from 3.1 to 4.0, which is close to the PFHxS:PFOS ratio in the house dust (2.7), family room carpet (2.5) and dining room carpet (3.1). No other Scotchgard™ formulations that we are aware of, including the 6 others analyzed by 3M Co. *(3M Company. 2000),* contain more PFHxS than PFOS, thus it is likely that one of these two products was applied to the current home on at least one occasion. Interestingly, these two formulations contain non-detectable levels of N-MeFOSE and other related PFOS-precursors, except for traces of perfluorooctanesulfonamide (FOSA) (6 ppm maximum), which are the conventional building blocks of 3M's carpet treatment formulations.[37]

After PFOS production stopped in 2002, PFHxS was used as a substitute for PFOS for impregnation of carpets *(Stockholm Convention. 2009, National Institutes of Health. 2011).* Aside from our current study, other evidence is emerging that the increasing use of PFHxS has impacted human and environmental exposure. *Goosey et al. (2011)* detected high PFHxS:PFOS ratios

in indoor and outdoor air samples collected in the UK in 2008/2009 and higher PFHxS outdoor air concentrations relative to 2005. *Glynn et al. (2012)* reported that PFHxS concentrations among primiparous women in Sweden have increased at a rate of 8.3%/yr. since 2000, and that PFHxS concentrations were similar to PFOS by 2010. However, based on Swedish pooled human milk samples collected during the period 1972 to 2008, *Sundstrom et al. (2011)* found a decreasing trend of all three major PFAS, namely PFOS, PFOA and PFHxS.

Based on the absence of any PFHxS precursors in the gas-phase of the air samples, it is likely that the main pathway of PFHxS intake among the family members was through ingestion of house dust contaminated with PFHxS from the house carpet. The partitioning or movement of PFASs from carpet into house dust may have been accelerated by the in-floor heating system underlying the carpet, but this remains uncertain. The high serum concentrations among the 3 youngest children support a household dust source, as children can ingest up to 200 mg of dust daily, compared to approximately 50 mg for adults *(US EPA. 2008).* Direct contact of children's hands on the carpets, and subsequent ingestion or dermal absorption cannot be ruled out as contributing to the exposure. In a recent Canadian study, *Shoeib et al.(2011)* showed that ingestion of house dust by toddlers was the main route of intake for PFOS and PFOA, and *Egeghy et al. (2011)* suggested that dust ingestion may be the most important pathway for PFOS intake in children. It is only germane to note that for other persistent organic pollutants such as polybrominated diphenyl ethers *(Fischer et al. 2006)* and hexabromocyclododecanes *(Roosens et al. 2009)* that house dust

ingestion/inhalation is an important pathway of exposure. However, caution must be exercised not to extrapolate the findings of this case study to the general population, as it is clearly documented that dietary exposure is the main exposure pathway for the general population, especially for adults.

Despite that many publications already point to the fact that one of the principal uses of PFASs was on carpets as dirt repellents, to our knowledge this is the first study showing elevated blood levels of PFASs in members of a family where such material had been documented to have been applied to their house carpet. From this case-study it is not clear how the high Scotchgard™ application frequency, the application of an unusual Scotchgard™ formulation containing elevated PFHxS and PFOS, or the unusual home characteristics (i.e. heated floors, no fresh-air intake) contributed to the overall high exposure, but some evidence suggests that the magnitude of exposure may not be unprecedented, and that other Canadian families may have similar exposure. For example, in a small sample (*n*=67) of Canadian households *Kubwabo et al. (2005)* reported a maximum house dust PFHxS concentration (4305 ng/g) that was higher than in the current study. Furthermore, in light of the current findings for this high socioeconomic status family, and the results of *Nelson et al. (2012),* wherein statistically significant associations between family income and serum PFAS levels were reported for the 2003-2006 NHANES cohort, we believe it is worthwhile that future biomonitoring studies pay particular attention to children and pregnant women in this high-exposure risk group, not only for PFASs but also in the broader context of indoor exposure to environmental toxicants.

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CHAPTER 4: Relative Binding Affinities of PFOS and PFOA Isomers for Serum Proteins

Sanjay Beesoon and Jonathan W Martin

Abstract

Perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) are the most prominent perfluoroalkyl substances (PFAS) in humans and umbilical cord blood. Historically, PFOS and PFOA were manufactured and sold as technical mixtures of linear and branched isomers. Biomonitoring studies have shown unexpectedly high proportions of branched PFOS isomers in human serum. In pregnant women, the transplacental transfer efficiencies (TTEs) of branched PFOS and PFOA isomers are higher than the corresponding linear isomer, but mechanisms controlling the disposition of the isomers have not been studied. Here, binding affinity assays are conducted using ultrafiltration devices to examine the dissociation constants of pure PFOS and PFOA isomers with human serum albumin (0.006 M), and furthermore to study the behaviour of technical mixtures incubated with both calf and human serum samples. Dissociation constants (K_d) showed that linear PFOS and PFOA were more strongly bound to human serum albumin than the corresponding branched isomers. K_d for linear PFOS (8.16 (±3.53) x 10^{-8} M) was at least three orders of magnitude lower than its respective branched isomers (3.58 (±1.53) x 10^{-4} M for 3*m*-PFOS, 7.83 (±1.02) x 10^{-5} M for 4*m*-PFOS and 9.10 (±4.87) x 10^{-5} M for 5*m*-PFOS). For PFOA, K_d for the linear isomer (1.32 (±0.91) x 10^{-4} M) was two orders of magnitude lower than for 4*m*-PFOA (2.82 (±1.61) x 10^{-2} M), but only three times smaller than for 3*m*-PFOA (4.19

(±2.40) x 10^{-4} M), and approximately half the estimate for 5*m*-PFOA (3.0 (±2.25) x 10^{-4} M). Furthermore, when human or calf serum were spiked with technical mixture of linear and branched isomers and subjected to ultrafiltration, the percentages of branched isomers in the ultrafiltrates were enriched. These data provide logical explanations for the higher placental transfer efficiencies, and preferential renal clearance of branched isomers as observed in multiple animal and human studies.

4.1 Introduction

As a result of their historical production *(Paul et al. 2009; Prevedouros et al. 2006)* and usage in multiple consumer products and industrial applications over the past 60 years, perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) are among the most prominent xenobiotics detected in human blood *(Centers for Disease Control and Prevention 2013; Health Canada 2010; Karrman et al. 2006); (Kannan et al. 2004)* and in environmental samples including house dust *(Awasum Bjorklund et al. 2009; Harrad et al. 2010; Kato et al. 2009),* ocean waters *(Ahrens et al. 2010; Martin et al. 2004; Stock et al. 2007),* lakes *(Martin et al. 2004; Stock et al. 2003)* and in wild-life *(Giesy and Kannan 2001).* PFOS and PFOA are persistent in the environment, can bioconcentrate in fish and biomagnify in foodwebs *(Martin et al. 2003; Martin et al. 2004)*. Based on animal toxicity studies, they have also been shown to have a range of toxic effects including neurotoxicity (*Butenhoff et al. 2009; Chen et al. 2013; Kawamoto et al. 2011; Mariussen 2012),* developmental toxicity *(Lau et al. 2004b; US EPA 2003),* carcinogenicity *(3M Company 1987; Benninghoff et al. 2012; Bjork et al. 2011; Butenhoff et al. 2012; Elcombe et al. 2012; Hu and Hu 2009; Qazi et al. 2010; Renner 2001),*

hepatotoxicity *(Kennedy et al. 2004; Lau et al. 2007; OECD 2002)* and may also disrupt the endocrine system *(Du et al. 2012; Gao et al. 2012; Knox et al. 2011; Liu et al. 2007; Spachmo and Arukwe 2012; White et al. 2011).*

The main historical manufacturer of PFOS and PFOA, the 3M Company, voluntarily phased out PFOS and PFOA production between years 2000 and 2002 *(USEPA 2000)* after their wide human *(Hansen et al. 2001)* and environmental distribution *(Giesy and Kannan 2001)* was first reported. Prior to this, the 3M Company manufactured PFOS and PFOA for over 4 decades by an industrial process known as electrochemical fluorination (ECF). This process yielded a consistent mixture of branched and linear isomers in resulting commercial products. For PFOS the composition was approximately 70% linear and 30 % branched, while for PFOA the composition was approximately 80% linear and 20 % branched *(Reagen 2007).*

Two studies have compared the relative toxicities of linear and branched isomers of PFOS or PFOA. In the first, *Loveless et al. (2006)* fed the ammonium salt of PFOA (APFO) to rats and mice in one of three following formulas: pure linear APFO, pure branched APFO, or a mixture of linear and branched APFO (ratio of 78:22), and monitored toxicological endpoints such as lipid profiles, liver and kidney weights, loss or gain in body weights and hepatic peroxisomal β-oxidation. Compared to control rats and mice, animals receiving 30 mg/kg pure linear APFO, and linear/branched APFO, had significantly lower weight gain over a 13 day period, while those receiving pure branched APFO had normal weight gain; a statistically significant decrease in total cholesterol, HDL-cholesterol, non HDL-cholesterol and triglycerides was noted for all three

APFO treatments. The authors came to the conclusion that branched isomers of APFO were less toxic than the linear isomer, based on overall results that pure branched APFO treatment had the least adverse effect on body weights, food intake and efficiency, hepatic peroxisomal β-oxidation and organ weights. Whether the lower toxicity of branched isomers is related to their faster elimination from the rodent's circulation or to their molecular interaction with receptors is still debatable. In the second study, *O'Brien et al. (2011a)* used microarray technology to compare the effects of technical PFOS (a mixture of linear (60-70%) and branched PFOS (30-40%)), versus pure linear PFOS, on the transcriptional profiles of chicken embryonic hepatocyte (CEH) cultures in-vitro. At equivalent 10 μM concentrations, technical PFOS upregulated 278 genes, compared to only 104 genes for pure linear PFOS, and downregulated 62 genes, compared to only 26 genes for pure linear PFOS. The very limited information available for PFOS must be cautiously interpreted, but it suggests that the branched isomers may induce different effects than linear PFOS, which may potentially translate into different toxicities.

PFOS and/or PFOA isomer specific disposition has been studied in fish *(Sharpe et al. 2010),* chicken eggs *(O'Brien et al. 2011b),* rodents *(Benskin et al. 2009a; De Silva et al. 2009)* and in humans *(Zhang et al. 2013).* In rainbow trout and zebrafish *(Sharpe et al. 2010*), preferential bioaccumulation of linear PFOS was shown in fish tissues compared to all branched isomers. Chicken eggs injected (in the air cell) with technical PFOS had higher bioaccumulation of linear PFOS in the embryonic liver tissue compared to the branched isomers *(O'Brien et al. 2011b).* In Sprague Dawley rats *(Benskin et al. 2009a; De Silva et al. 2009)* exposed orally to technical mixtures of PFOS and PFOA, the proportion of branched isomers in the urine (the primary mode

of excretion) was higher than in the blood or the corresponding technical mixture, also indicating a preference to accumulate the linear isomer over branched isomers. The only study to date on the isomer-specific elimination of PFOS or PFOA in humans also showed a consistent preferential urinary excretion of branched isomers in a Chinese population *(Zhang et al. 2013).* In this pharmacokinetic context, it is therefore interesting to note that human blood is often enriched in branched PFOS isomers (i.e. >30% branched PFOS) compared to historical ECF products *(Beesoon et al. 2011; Kärrman et al. 2005),* as this cannot be explained by any of the above information suggesting that the linear isomer is the most accumulative.

PFOS and PFOA isomers can cross the human placenta and enter into fetal circulation, and three studies have shown the preferential enrichment of branched PFOA and PFOS isomers in cord blood, relative to maternal blood *(Beesoon et al. 2011; Gützkow et al. 2012; Hanssen et al. 2010b).* The higher transplacental transfer efficiency of branched PFOS and PFOA is rather surprising. First, because more hydrophobic molecules are generally more efficient at crossing the lipid bilayer of endothelial cells *(Plonait and Heinz 2011),* and based on their elution orders in reversed phase chromatography *(Benskin et al. 2007b),* branched PFOS and PFOA isomers are less hydrophobic than the linear isomers. Secondly, as predicted by Rama Sastry *(Rama Sastry 1995; Rama Sastry 1999)* the more "rounded" a molecule is, the less efficient it will be at crossing the placental barrier; thus when the geometry of the branched isomers (a higher cross sectional diameter) are compared to their linear counterparts, the transplacental movement of the branched isomers would be predicted to be less than the corresponding linear isomers.

In a direct binding assay, conducted at a technical PFOS concentration of 2.35 mg/mL, with bovine serum albumin (1 mg/mL), and detection of the albumin-bound fraction of PFOS by quadrupole time of flight mass-spectrometry, *Jones et al. (2003)* reported that nearly all (> 98%) PFOS was bound to bovine albumin. Using equilibrium dialysis, *Zhang et al. (2009)* later concluded that 1 molecule of serum albumin can bind up to 45 molecules of PFOS non-covalently. For PFOA, *Han et al. (2003)* used size exclusion chromatography and ligand blotting methods to determine that over 90% of PFOA is bound to albumin in human or rat blood under normal physiological conditions. *Bischel et al. (2010; 2011)* used equilibrium dialysis with 200 µM bovine serum albumin and reported that over 99% of PFOS (0.013 µM to 0.260 µM) and PFOA (0.023 µM to 0.320 µM) were non-covalently bound to the albumin. To date, no studies have examined the isomer specific binding of PFOS or PFOA, even though *Jones et al. (2003)* long ago suggested that the "*consequences of differential binding of straight-chain and branch-chain forms of PFOS to albumin require further investigation"*.

Here we hypothesized that the binding affinities of linear PFOS and PFOA to serum proteins would be higher than any corresponding branched isomer. If true, then under physiological conditions this would leave a higher proportion of unbound branched isomers in the serum to cross the placental barrier, or to be filtered through the glomerular membrane and excreted in urine; both of which have been observed to occur in humans. To test this hypothesis, an ultrafiltration method was employed to estimate the dissociation constants (K_d) of linear and 3 individual branched isomers (3*m*, 4*m* and 5*m*) of PFOS and PFOA with human serum albumin,

and to study the behaviour of technical PFOS and PFOA mixtures spiked in whole calf and human serum.

4.2 Materials and Methods

4.2.1 Chemical standards and reagents:

The same nomenclature proposed by *Benskin et al. (2007a)* is used here for the individual branched PFOS and PFOA isomers. The structures of linear and branched isomers used here are also shown in Figure 4.1. Linear PFOS (50 µg/mL), linear PFOA (50 µg/mL) and mass-labeled internal standards (MPFAC-MXA) were obtained from Wellington Laboratories (Guelph, ON, Canada). Technical mixtures of PFOS (Br-PFOS) and PFOA (T-PFOA) were also purchased from Wellington Laboratories. Exceptionally high concentrations of individual isomers such as 3*m*-PFOS (23.33 µg/mL), 3*m*-PFOA (44.40 µg/mL), 4*m*-PFOS (26.39 µg/mL) 4*m*-PFOA (58.00 µg/mL), 5*m*-PFOS (42.83 µg/mL), 5*m*-PFOA (84.00 µg/mL), which are not commercially available, were prepared and donated by Wellington Laboratories on a one-time basis (Guelph, ON, Canada). Fatty acid and globulin free human serum albumin (HSA; purity ≥ 99 % by agarose gel electrophoresis) was purchased as a lyophilized powder from Sigma Aldrich (Oakville, ON, Canada). All chemicals used in the preparation of organic and aqueous mobile phases (methanol, water, formic acid and ammonium hydroxide) for liquid chromatography were of HPLC grade and purchased from Fisher Scientific (Ottawa, ON, Canada).

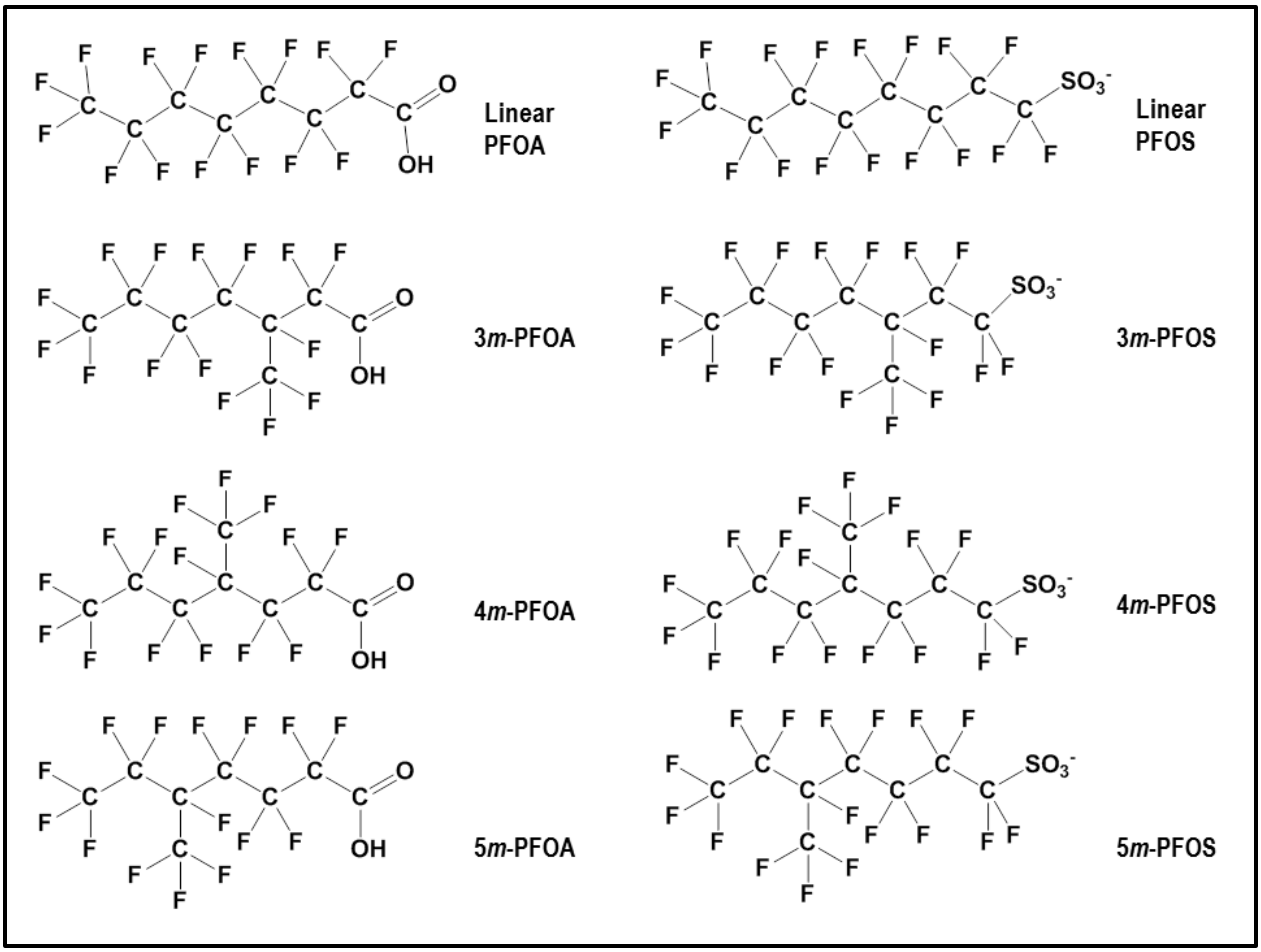

Figure 4.1. Structures of linear and branched PFOA and PFOS isomers for which dissociation constants were calculated.

4.2.2 Incubation and ultrafiltration of specific isomers with Human Serum Albumin (HSA) and ultrafiltration

HSA (0.006 mM) was prepared in HPLC grade water and 900 - 1000 µL aliquots were dispensed into 1.5 mL Eppendorf™ safe-lock polypropylene microcentrifuge tubes. This particular concentration of HSA was chosen such that it falls within the range of PFOS and PFOA (both linear and branched) concentrations used in this experiment, thus a 1:1 PFOS (or PFOA) molar ratio was achieved at a given point within the range of selected concentrations. The albumin solutions were then spiked with pure linear or branched isomer standards such that the total volume reached were 1000 µL in all tubes. The concentrations of individual PFOS and PFOA isomers ranged from 0.002 mM to 0.012 mM. A series of quality control samples (containing no protein) were prepared with pure HPLC water spiked with different concentrations of PFOS or PFOA to examine for any non-specific binding of PFOS or PFOA to the Centrifree® ultrafiltration (UF) device membrane, or to the walls of the tubes. All tubes were mixed by gentle inversion, to avoid frothing and possible denaturing of the albumin *(Clarkson et al. 1999),* and were incubated at 37°C in a circulating water bath (BÜCHI Labortechnik GmbH, Flawil, Switzerland) for 1 hour, after which 700 µL was transferred by pipette to UF device (Centrifree®), taking care to avoid air bubbles. These devices accommodated a maximum volume of 1 mL and the molecular weight membrane cut-off was 30,000 Daltons. After loading the UF devices, they were centrifuged at 1500 g at 37°C for 30 minutes (Sorvall, ST 40 R, Thermo Fisher Scientific, Ottawa, Canada). The remaining 300 µL of incubated solution (not centrifuged – thus representing total PFOS and PFOA) and the ultrafiltrates were kept at -20° C

pending sample preparation and analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS).

4.2.3 Incubation and ultrafiltration of technical PFOS and PFOA mixtures in whole calf and diluted human serum

One mL aliquots of whole calf serum (Lampire Biological Laboratories Inc., Pipersville, PA 18947, USA) were spiked with 100 ng/mL, 200 ng/mL and 300 ng/mL of technical PFOS (78.8 % linear) and T-PFOA (79.0 % linear) respectively. One mL aliquots of diluted (1 in 10) human serum (Lampire Biological Laboratories Inc., Pipersville, PA 18947, USA) were spiked at low mg/L ECF-PFOS (gift from 3M Laboratories) and T-PFOA (purchased from Wellington Laboratories, Guelph, ON, Canada). Unlike the dissociation constant experiment described above, whereby pure individual isomers were spiked into pure human serum albumin, in this case the whole serum (containing all endogenous proteins in equilibrium with their endogenous substrates) was spiked with a mixture of linear and branched isomers. The reason to dilute the human serum sample was three fold: first, this diminished the background signal PFOS or PFOA isomers in the serum. Second, given that the serum sample was a commercial one (purchased from Lampire Laboratories, Pipersville, PA, USA) and most likely from non-fasting individuals, it was visibly turbid. Third, since the spiking concentrations of PFOS and PFOA was in the low mg/L range, by diluting the human serum sample tenfold, the expectation is that the albumin concentration in the serum would match the spiking concentrations of the ECF PFOS (5.0 to 60 mg/L) and T-PFOA (0.5 to 3.0 mg/L)

4.2.3 Sample preparation:

Prior to analysis, uncentrifuged samples, representing total (free + bound) PFOS or PFOA, were diluted 100-fold with methanol, while the ultrafiltrates were diluted 20-fold with methanol, and a portion of these were spiked with 5 µL of 100 ng/mL of a mixture of mass-labelled internal standards (MPFAC-MXA). All samples were then centrifuged at 14,000 revolutions per minute (Thermo IEC Micromax RF, Thermo Fisher Scientific, Ottawa, Canada) for 30 minutes and the supernatants were then transferred to HPLC vials before analysis.

4.2.4 LC-MS/MS Conditions

The method of *Benskin et al. (2007b*) was adapted to identify and quantify the individual linear and branched isomers of PFOS and PFOA in the samples. Chromatographic separation of linear and branched isomers was performed using a FluoroSep RP Octyl column (3µ 100Å, 15 cm x 2.1 mm, ES Industries, West Berlin, NJ) at a flow rate of 150 µL/min on a Shimadzu UFLC-XR (Kyoto, Japan). A gradient elution method was used with mobile phases *A*: 5 mM ammonium formate at pH 4.0, and *B*: methanol. Initial conditions (60% A and 40% B) were held for 0.3 min, ramped to 55 % B by 4.0 min, increased to 80 % B by 20 min, 100 % B by 22 min and held until 25 min. Mass spectral data were collected on a triple-quadrupole mass spectrometer (API 4000, AB Sciex, Concord, ON, Canada) using electrospray ionization operated in negative ion mode. The following multiple reaction monitoring transitions were used to quantify the ions representing each isomer: Linear PFOS: *m/z* 499/80, 3*m*-PFOS: *m/z* 499/130, 4*m*-PFOS: *m/z* 499/330, 5*m*-PFOS: *m/z* 499/130, Linear PFOA : *m/z* 413/369, 3*m*-PFOA: m/z 413/169, 4*m*-PFOA: *m/z* 413/119 and 5*m*-PFOA: *m/z* 413/219.

4.2.5 Derivation of isomer-specific dissociation constants (K_d)

For all samples (blanks, quality controls, filtrates and uncentrifuged) the relative response (RR) of each individual PFOS or PFOA isomer was calculated relative to linear ^{13}C-labelled PFOS or PFOA internal standard, respectively. The percentage of free PFOS or PFOA isomer in a sample was calculated as in equation (1), using linear PFOS as the example:

$$\%Free_{linearPFOS} = \frac{RR_{UF}}{RR_{UC}} \times 100 \quad (1)$$

where RR_{UF} represents the calculated relative response of linear PFOS in the ultrafiltrate (i.e. free linear PFOS) and RR_{UC} represents the relative response of linear PFOS in the uncentrifuged sample (i.e. free and albumin-bound linear PFOS). From equation 1 above, the concentration of free linear PFOS ($CFree_{linearPFOS}$) and bound linear PFOS ($CBound_{linearPFOS}$) at a given spiked concentration of linear PFOS in 0.006 mM albumin can be calculated according to equations (2) and (3), respectively.

$$CFree_{linearPFOS} = \frac{\%Free_{linearPFOS}}{100} \times TotalConc_{linearPFOS} \quad (2)$$

$$CBound_{linearPFOS} = TotalConc_{linearPFOS} - CFree_{linearPFOS} \quad (3)$$

In the current study, different free ligands (L_F) (i.e. linear and branched isomers of PFOS and PFOA) over a range of concentrations bind reversibly to the protein receptor (R) at a fixed concentration (i.e. 0.006 mM human serum albumin) to form a complex (LR), as in equation (4).

$$L_F + R \underset{K_{off}}{\overset{K_{on}}{\Longleftrightarrow}} LR \qquad (4)$$

At equilibrium, the rate of the forward reaction (V_f), where $V_f = k_{on}\ [L]\ [R]$ is equal to the reverse reaction (V_b), where $V_b = k_{off}\ [LR]$ (equation 5), which can be rearranged to solve for k_{-1}/k_1, also known as the dissociation constant (K_d) (equation 6).

$$K_{on}\ [L_F]\ [R] = K_{off}\ [LR] \qquad (5)$$

$$\frac{[L_F]\ [R]}{[LR]} = \frac{K_{off}}{K_{on}} = K_d \qquad (6)$$

Thus, K_d is a measure of the relative binding affinity between L and R, with high values of K_d indicating low binding affinities, and low values of K_d indicating high binding affinities. To experimentally derive an estimate for the binding between PFOA and rat or human serum proteins, (Han et al. (2003) formerly proposed the following variant of Scatchard equation linking L_F to K_d:

$$\frac{[L]_F}{r} = \frac{[L]_F}{n} + \frac{K_d}{n}$$

Where $[L]_F$ is the concentration of unbound ligand, r is the moles of ligand bound per mole of albumin, n is the number of binding sites and K_d is the dissociation constant. A plot of $\frac{[L]_F}{r}$ versus $[L]_F$ is a straight line with an intercept of $\frac{K_d}{n}$ and a slope of $\frac{1}{n}$.

4.3 Results and Discussion

4.3.1 Dissociation constants of linear PFOS and PFOA with human serum albumin

Pure water spiked at different concentrations of linear and branched PFOS and PFOA and centrifuged through the UF device showed recoveries ranging from 87 % to 103 %, and there was no significant binding of any isomer to the filter membrane or walls of the UF device. The dissociation constant of linear PFOS *($K_d = 8.16 \times 10^{-8}$ M)* was determined to be much lower than that of linear PFOA *($K_d = 1.32 \times 10^{-4}$ M)* indicating a much stronger binding affinity of PFOS to HSA than PFOA; although there were 2 binding sites on the human serum albumin molecule for both linear PFOS and linear PFOA. This general finding is consistent with the findings of *Salvalaglio et al. (2010)* who reported that a higher percentage of PFOS (98 %) was bound to HSA than PFOA (95%); based on computer models that used a combination of docking and molecular dynamics simulation. It is only germane to note that at the highest spiked linear PFOS concentration used here (0.012 mM), that the ratio of PFOS to HSA was also 2, thus all experimental data should be valid, and saturation of the albumin receptor did not occur.

Although other previous evidence exists that PFOS indeed binds to HSA *(Luo et al. 2012; Zhang et al. 2009),* only *Chen and Guo (2009)* have reported a quantitative estimate of the dissociation constant for PFOS (purchased from Sigma Aldrich, likely an electrochemical mixture of isomers) based on a site specific fluorescence analytical method and HSA: $2.2 \pm 0.43 \times 10^{-4}$ M at the tryptophan site, and $7.6 \pm 0.3 \times 10^{-6}$ M at the Sudlow binding site II. As far as the interaction of PFOA with HSA is concerned, there is great variability in the formerly reported K_d's. For example, *Wu et al. (2009)* reported a K_d of 2.5×10^{-4} M based on isothermal titration

calorimetry, while a value of 3.1 x 10^{-4} M was generated using equilibrium dialysis. *Han et al. (2012)* reported a K_d of 2.6 x 10^{-3} M using size exclusion chromatography, and a similar value of 3.5 x 10^{-3} M using nuclear magnetic resonance. A fluorescence method was used in two independent studies of PFOA, and the reported K_d values differed by an order of magnitude: 2.2 x 10^{-4} M *(Hebert and MacManus-Spencer 2010)* and 2.7 x 10^{-5} M *(Chen and Guo 2009).* The widely variable dissociation constants for PFOA may be due to methodological differences, but more importantly might be due to the source, and thus isomeric purity, of the PFOA being used.

4.3.2 Dissociation constants of branched PFOS and PFOA

K_d calculation for the isomers of PFOS (Figure 4.2) and PFOA (Figure 4.3) showed that linear isomers associated more strongly with albumin than any of the branched isomers, and that the effect was much more pronounced for PFOS isomers. Furthermore, the calculated number of binding sites on the albumin molecule was close to 2 for both linear-PFOS and linear PFOA, but less than 1 for all of the branched isomers investigated. For 4*m*-PFOA, binding was quite weak and no statistically significant regression was found, (p-value >0.05), thus the K_d and number of binding sites are not reported.

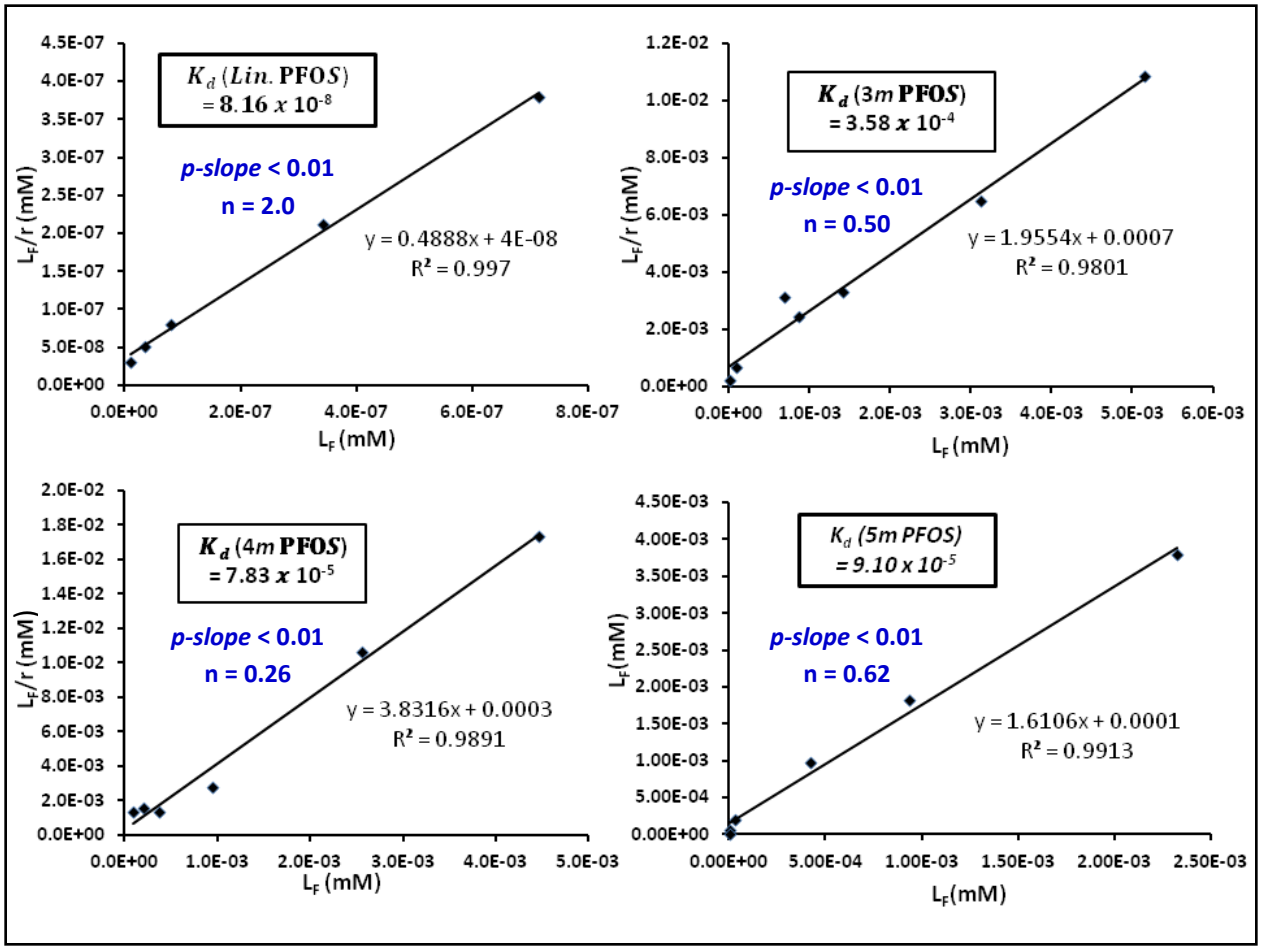

Figure 4.2. Scatchard plots used to calculate dissociation constants (M) of linear and three individual branched isomers of PFOS. The p-value is less than 0.01 in all cases meaning that the calculated slopes of the regression lines are all statistically significantly different from zero at the 99% confidence interval. The "n" values represent the number of binding sites on the albumin molecule for that specific isomer - for example there are 2 binding sites for linear isomer, but less than one for the branched isomers.

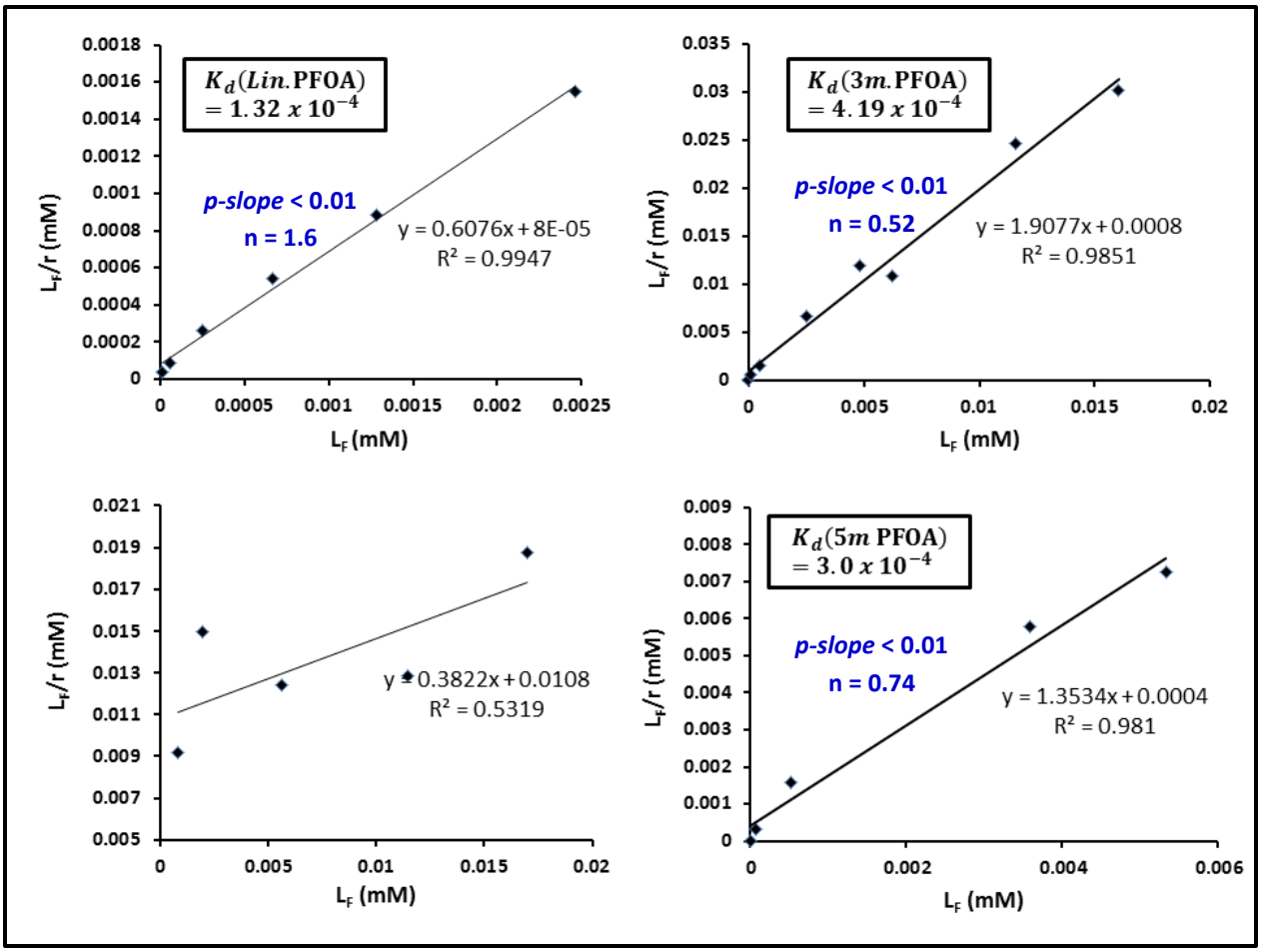

Figure 4.3. Scatchard plots used to calculate dissociation constants (M) of linear and three individual branched isomers of PFOA. The p-value is less than 0.01 for linear-PFOA, 3m-PFOA and 5m-PFOA, meaning that the calculated slopes of the regression lines are statistically significantly different from zero at the 99% confidence interval. P-value for 4m-PFOA > 0.05, meaning that the slope of the regression line is NOT significantly different from 0. The "n" values represent the number of binding sites on the albumin molecule for that specific isomer- for example there are 2 binding sites for linear isomer, but less than one for 3m-PFOA and 5m-PFOA. The K_d and n for 4m-PFOA could not be determined, due to very weak binding and no significant regression.

For PFOS, the K_d (± SE) of the linear isomer 8.16(± 3.53) x 10^{-8} M was at least three orders of magnitude lower than for any of its respective branched isomers: 3.58(± 1.53) x 10^{-4} M for 3*m*-PFOS, 7.83(± 1.02) x 10^{-5} M for 4*m*-PFOS and 9.10(± 4.87) x 10^{-5} M for 5*m*-PFOS. For PFOA,

the K_d of the linear isomer 1.32(± 0.91) x 10^{-4} M was only approximately three times smaller than for 3*m*-PFOA 4.19(± 2.40) x 10^{-4} M, and approximately half the estimate for 5*m*-PFOA 3.0 (± 2.25) x 10^{-4} M. For 4*m*-PFOA the K_d could not be determined owing to a non-significant regression (Figure 4.3), likely because the binding is much weaker than for the other isomers.

4.3.3 Isomer Specific Binding in Calf Serum

To determine if the general relationships discovered for binding to pure HSA would also be true in real serum, calf serum was spiked at three concentrations of technical PFOS or PFOA, allowed to equilibrate, and then centrifuged in the UF device. Linear PFOS bound more strongly to total proteins than the respective branched isomers, as the percentages of total branched PFOS (measured by the cumulative signal in the *m/z* 499/80 transition) and PFOA (measured by the cumulative signal in the *m/z* 413/369 transition) isomers were consistently and significantly (p-value=0.001) higher in the ultrafiltrates compared to the PFOS spiked calf serum prior to ultrafiltration (Figure 4.4). For PFOA, statistically significant higher percentages of branched isomers were detected (p-value=0.02) in the ultrafiltrates at spiking levels of 200 ng/mL and 300 ng/mL. At 100 ng/mL, the ratio of % branched PFOA isomers in the ultrafiltrate could not be determined, owing to background signal of linear PFOA in the calf serum.

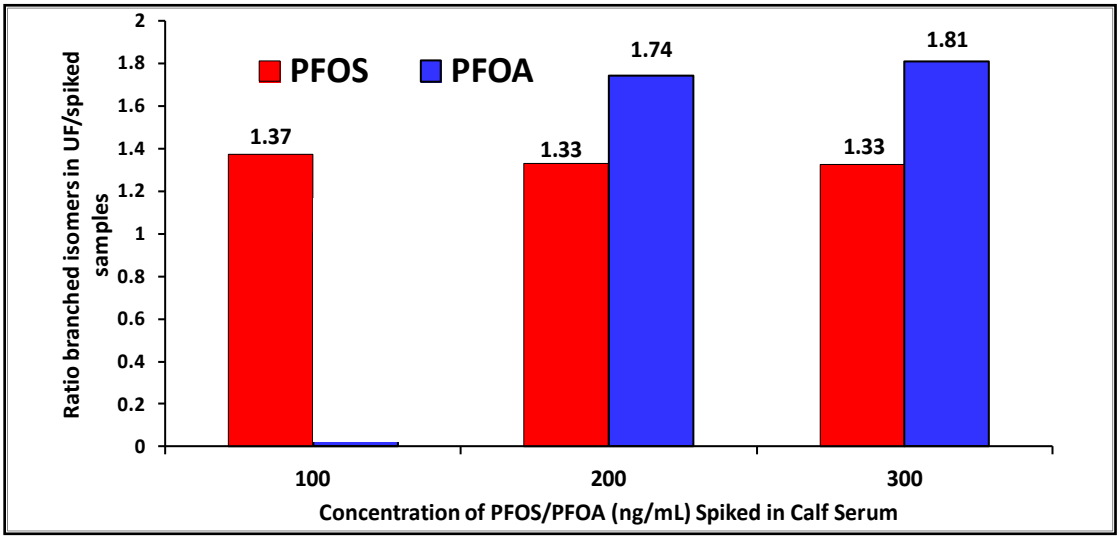

Figure 4.4. Mean ratio of the percentage of branched PFOS and PFOA isomers in ultrafiltrates compared to technical PFOA- and PFOS-spiked calf serum at 100 ng/mL, 200 ng/mL and 300 ng/mL. Values greater than 1.0 indicate an enrichment of branched isomers in the ultrafiltrate, compared to spiked serum. Experiments were conducted in duplicates at each spiked concentration.

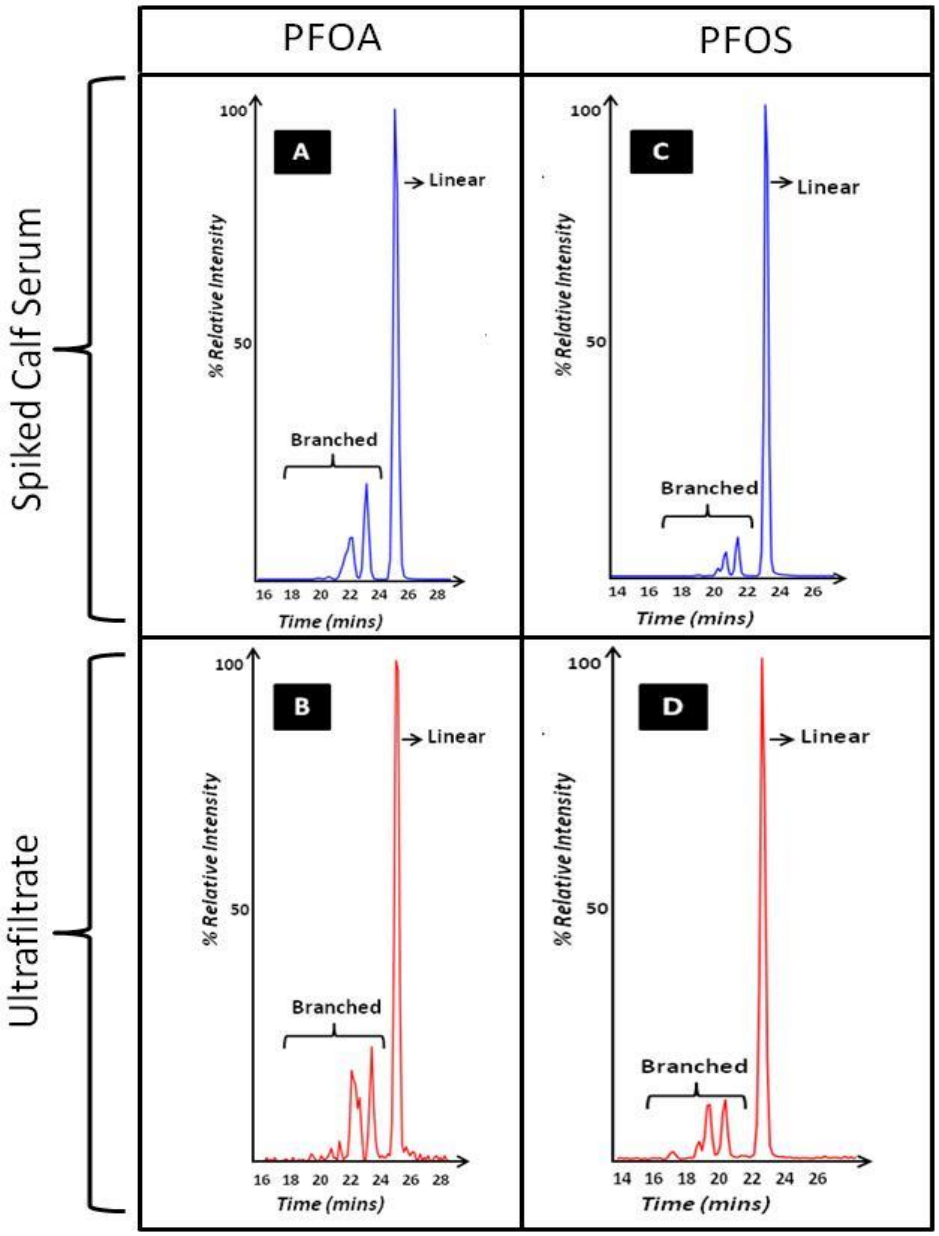

Figure 4.5. Chromatograms showing relative amounts of linear and branched isomers of PFOA (A and B) and PFOS (C and D) in spiked calf sera (A and C) and the associated ultrafiltrates (B and D). Both calf serum samples were spiked at 300 ng/mL with technical PFOA or PFOS. Note the higher relative amounts of branched isomers in the ultrafiltrate samples, particularly for earlier eluting branched isomers. All the chromatograms have been normalized (y-axis) with respect to response of the linear isomer.

The higher percentage of branched isomers of PFOS and PFOA in the ultrafiltrate can also be visualized by inspection of the chromatograms of spiked calf serum and the associated ultrafiltrates (Figure 4.5). From these chromatograms, it appeared that some of the earliest eluting branched isomers might be more enriched in the ultrafiltrate than the later eluting branched isomers. Thus, isomer specific data for PFOS (Figure 4.6) and PFOA (Figure 4.7) were examined for the spiked calf serum samples, using duplicate data for each of the three concentrations. This confirmed that each branched isomer was less strongly bound to calf serum proteins than the corresponding linear molecule, and also revealed some structural dependence. In fact, the percentage of the individual PFOS and PFOA mono-methyl branched isomers in the calf serum ultrafiltrates followed nearly the same structure activity relationship previously reported for human placental transfer of PFOS isomers whereby linear-PFOS had the lowest transplacental transfer efficiency (TTE) followed by *iso*-PFOS, 5*m*-PFOS, 4*m*-PFOS, 3*m*-PFOS, 1*m*-PFOS and the dimethyl-PFOS isomers *(Beesoon et al. 2011).* In the current experiment, with the exception of 1*m*-PFOS (Figure 4.6) and 3*m*-PFOA (Figure 4.7), as the perfluoro-methyl branching point moved closer to the acid functional group, the protein binding strength was reduced, and a higher percentage of the isomer was thus detected in the ultrafiltrates.

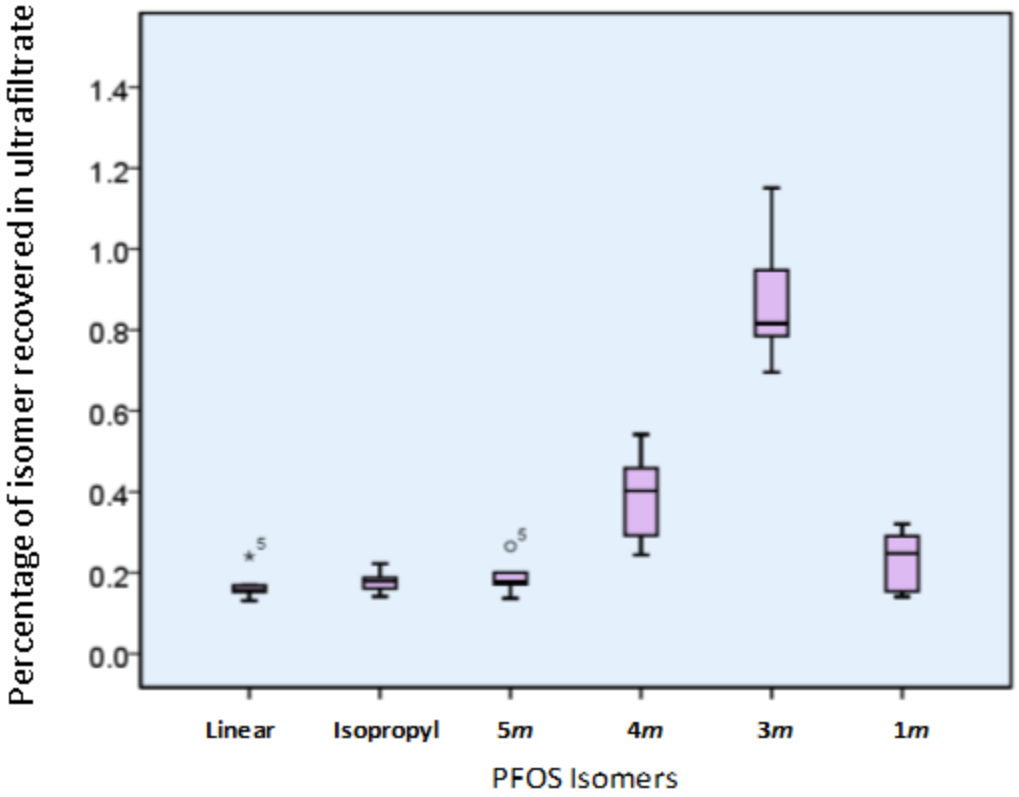

Figure 4.6. Box-Plot showing percentage of individual linear and branched isomers of PFOS in the spiked calf serum that was recovered in the ultrafiltrate. Using linear PFOS as an example, the data on the ordinate axis represent the percent of the total linear PFOS spiked in the calf serum that was recovered in the ultrafiltrate. The superscripts labelled "5" for the linear and 5m isomers are outliers.

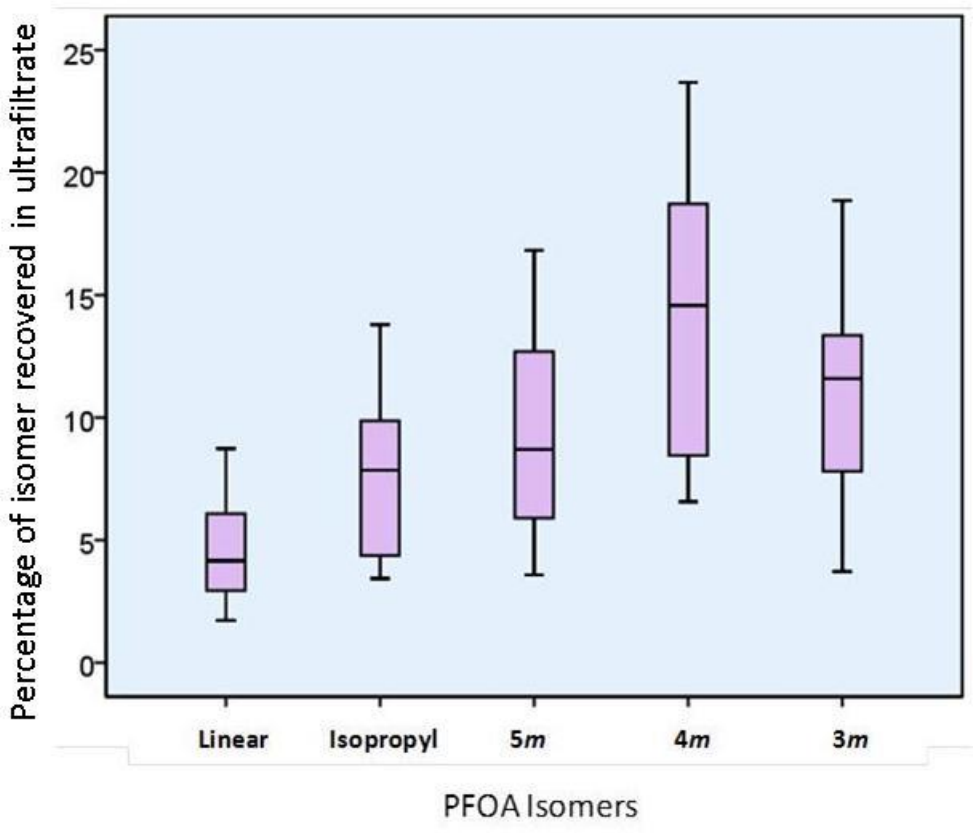

Figure 4.7. Box-Plot showing percentage of individual linear and branched isomers of PFOA in the spiked calf serum. Using linear PFOA as an example, the data on the ordinate axis represent the percent of the total linear PFOA spiked in the calf serum that was recovered in the ultrafiltrate.

4.3.4 Isomer Specific Binding in Human Pregnant Female Serum

Analysis of the percentage of branched PFOS isomers in the diluted (1 in 10 with 0.85 % sterile physiological saline) human serum samples spiked with varying concentrations (0 to 60 mg/L) of ECF PFOS show a consistently higher percentage of branched isomers in the ultrafiltrate compared to the unfiltered spiked samples. This is consistent with the calf serum data, whereby higher percentages of branched PFOS isomers were detected in the ultrafiltrate. This clearly

shows the higher binding affinities of branched PFOS isomers with human serum proteins, of which albumin is the major component. This finding is corroborated by the dissociation constant study which established that the dissociation constant of linear PFOS with pure HSA is four orders of magnitude lower than the three individual branched PFOS isomers tested, namely 3*m*-PFOS, 4*m*-PFOS and 5*m*-PFOS.

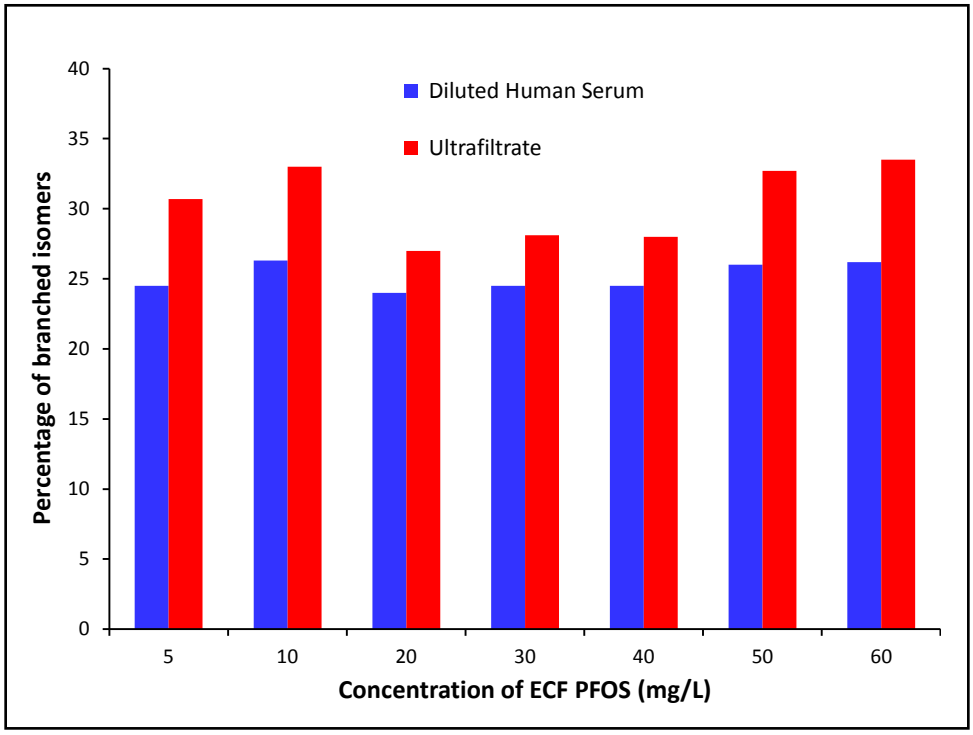

Figure 4.8. Percentage of branched PFOS isomers in ECF PFOS-spiked human pregnant female serum (blue bars) and the associated ultrafiltrates (red bars) over a range of concentrations. Given the high concentrations of ECF PFOS involved in this experiment and the scarcity of the latter chemical, only one replicate analysis for each concentration was performed.

Isomer specific PFOS analysis of spiked human serum samples and ultrafiltrates (Figure 4.9) showed, in general, that individual branched isomers (note that 3*m*- and 5*m*-PFOS were

integrated together due to poor resolution) had slightly lower binding affinities to the serum proteins compared to linear PFOS, but the results were less obvious than with calf serum (Figure 4.9). The exception to the general trend was 1*m*-PFOS, which appeared to be bound more tightly to serum proteins than any isomer (Figure 4.9).

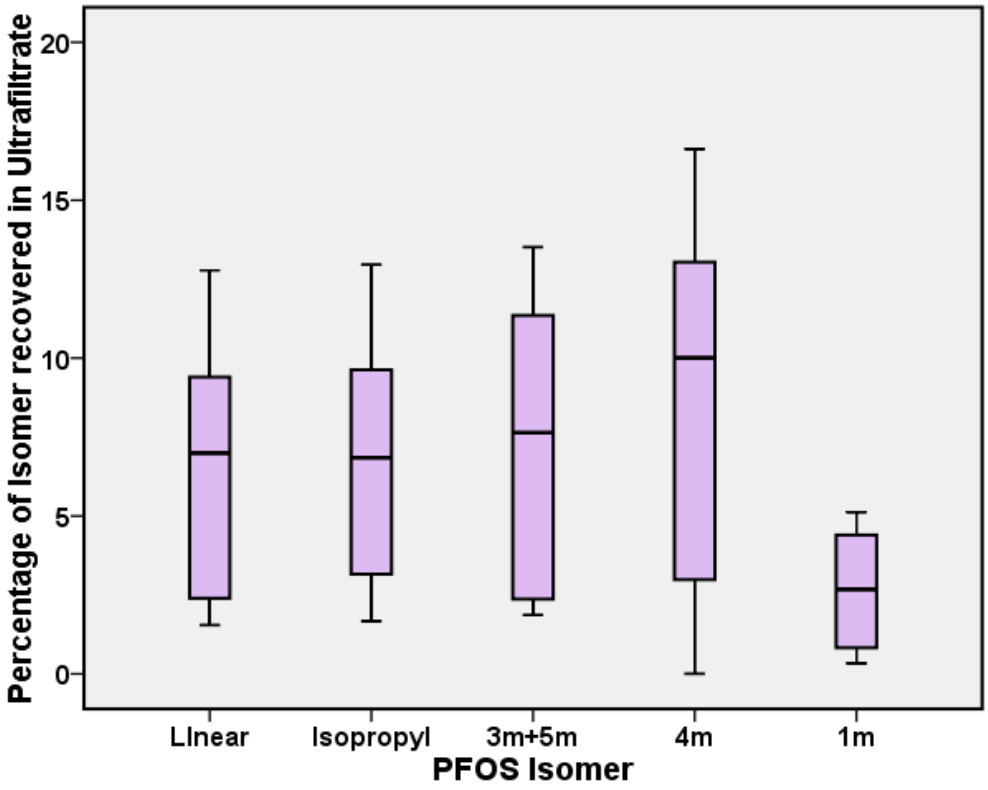

Figure 4.9. Box-Plot showing percentage of individual linear and branched isomers of PFOS in the spiked human serum that was recovered in the ultrafiltrate. Using linear PFOS as an example, the data on the ordinate axis represent the percent of the total linear PFOS spiked in the calf serum that was recovered in the ultrafiltrate.

To examine for any statistically significant differences in isomer specific binding affinities to human serum proteins, a univariate analysis of variance (2-way ANOVA) was conducted based on a general linear model equation followed by post hoc multiple comparisons for observed

means for each isomer (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). The ANOVA indicated a statistically significant isomer ($p < 0.001$) and concentration ($p < 0.001$) effect, and post-hoc comparisons indicated that the only statistically significant differences were for 1*m*-PFOS, whereby the percentage of unbound (free) molecules detected in the ultrafiltrate was significantly lower than any of the other isomers, namely linear PFOS (p-value: 0.03), iso-PFOS (p-value: 0.004), 3+5*m*-PFOS (p-value: 0.003) and 4*m*-PFOS (p-value< 0.001). This latter result may explain the exceptionally high biological half-life of 1*m*-PFOS compared to all other PFOS isomers (geometrical mean of 9.6 years in young Chinese females and 55 years in males) reported by Zhang et al. (2013). With its outstanding ability to bind to human serum proteins, it is expected that 1*m*-PFOS would not easily pass into the glomerular filtrate and be excreted through urine.

For PFOA, higher proportions of branched isomers, compared to linear PFOA, were also consistently detected in the female human serum ultrafiltrate at all six different spiking concentrations (0.5, 1.0. 1.5, 2.0, 2.5 and 3.0 mg/L). The mean percentage (95 % CI) of unbound (free fraction) linear PFOA, *iso*-PFOA, 5*m*-PFOA, 4*m*-PFOA, 3*m*-PFOA were 8.97 (6.63 – 11.3), 22.0 (9.94 – 34.1), 20.4 (14.9 – 25.9), 24.8 (18.4 – 31.2) and 23.1 (17.2 – 28.9) respectively. These data show a consistent trend whereby the binding affinities of the individual branched PFOA isomers to human serum proteins are consistently lower than that of linear PFOA. ANOVA, based on a general linear model equation showed a statistically significant isomer effect ($p < 0.001$), and pairwise comparisons showed that the higher unbound (free) fraction of the individual branched isomers compared to linear PFOA was statistically significant

in all cases ($P < 0.05$). As opposed to PFOS where no structure activity was seen, in the case of PFOA there was a clear structure activity relationship, showing that as the perfluoromethyl branching point moved closer to the carboxylate, the binding affinity to serum proteins decreased substantially (Figure 4.10).

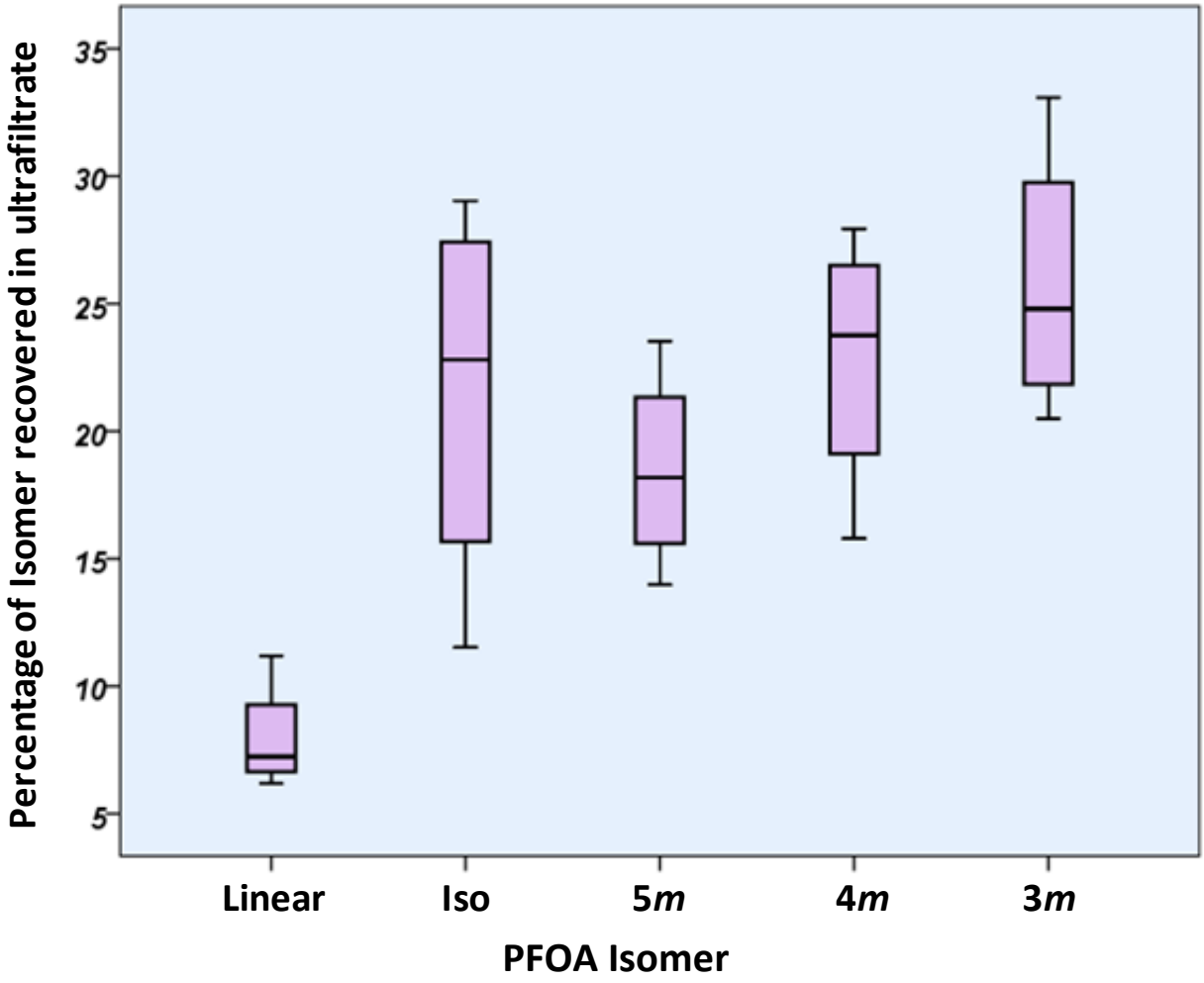

Figure 4.10. Box-Plot showing percentage of individual linear and branched isomers of PFOA in the spiked human serum that was recovered in the ultrafiltrate. Using linear PFOA as an example, the data on the ordinate axis represent the percent of the total linear PFOA spiked in the calf serum that was recovered in the ultrafiltrate.

4.3.5 Discussion and Conclusion

Previous studies have shown that the Centrifree® UF device is a quick, reliable and cost-effective method for measuring the free fraction of drugs or hormones in blood. For example, *Dow et al. (2006)* suggested the Centrifree® UF device as an excellent method for estimating the free fraction of pharmaceuticals in plasma, and elegantly derived the mathematical equation for calculating the percentage of plasma protein binding. In an attempt to estimate the bioavailability of triiodothyronine and thyroxine, *Gu et al. (2007)* used the Centrifree® UF device to separate bound and free fractions. More recently, *Jensen et al. (2011)* used the Centrifree® UF device to quantify the bound and free enantiomers of warfarin in human plasma, and the authors highlighted the exceptional reliability of the method.

Our findings on the higher binding affinities of linear PFOS and PFOA to serum albumin, relative to branched isomers, are consistent with various human and rodent pharmacokinetic data. For example, the higher transplacental transfer efficiencies of branched isomers of PFOS and PFOA reported in humans (Chapter 2) *(Beesoon et al. 2011; Gützkow et al. 2012; Hanssen et al. 2010a)* could be explained based on the fact that linear PFOS and PFOA are adsorbed most strongly to serum proteins, and thus are less bioavailable to cross the placental barrier. Furthermore, it is known that maternal albumin concentrations decrease through gestation, and that fetal serum albumin concentrations increase through gestation. For example, *Plonait and Heinz (2011)* reported fetal-maternal albumin ratios of 0.28, 0.66, 0.97 and 1.20 at 12-15, 16-25, 26-35 and 35-41 weeks of gestation respectively. Thus, the higher apparent transplacental transfer efficiencies (TTEs) of total PFOS and total PFOA determined at term, compared to

TTEs determined earlier in pregnancy (i.e. when the mothers blood sample is taken prior to term) *(Beesoon et al. 2011; Fei et al. 2007; Monroy et al. 2008)* could also be explained by the dynamics of albumin in maternal and fetal circulation. Moreover, the higher renal elimination of branched PFOS and PFOA isomers in people *(Zhang et al. 2012)* and rodents *(Benskin et al. 2009; De Silva et al. 2009)* is also consistent with the current data because as xenobiotics bind to serum albumin, or other large proteins, they cannot be filtered and eliminated in urine by the kidney. *D'Eon et al. (2010)* noted the exceptional binding of PFOA to HSA by NMR and postulated that the interactions of perfluorinated substances with albumin must have a direct bearing on elimination kinetics of these compounds from the human body; the current data support this proposition.

Using the theoretical concepts of ligand binding and a computer simulation strategy that integrates docking and molecular dynamics simulation data for the interaction between HSA and PFOS and PFOA, *Salvalaglio et al. (2010)* made a number of key observations: firstly, based on the free binding energies of PFOS-HSA and PFOA-HSA complexes at thermodynamic equilibrium, they reported a maximum number of 9 PFOA and 11 PFOS molecules that can be adsorbed on HSA at the critical micelle concentration (CMC), which the authors suggest as a possible explanation for the higher bioaccumulation potential of PFOS compared to PFOA. However, *Salvalaglio et al. (2010)* did not discriminate between linear and branched isomers in their theoretical computer modeling. Secondly, the authors suggest that the hydrophobic nature of the perfluoroalkyl chain plays a major role in the PFAA-HSA complex formation which favors bioaccumulation. Thirdly, the authors reported on the presence of interaction sites at the

core of the HSA molecule and that an increase in "size" of the ligand can reduce the ease of access to these sites because of steric hindrance. Given the more "rounded" structure of the branched PFOS and PFOA isomers compared to their linear counterparts, our findings on the lower binding affinities of branched isomers are in agreement with theoretical predictions of *Salvalaglio et al. (2010)*, that they are less likely to fit in the binding pockets of HSA. Furthermore, our data on the number of binding sites (n=2) on HSA for linear PFOS and PFOA, compared to the individual branched isomers ($n < 1.0$ in all cases, except for 4m-PFOA, but for which the n=2.6 is not statistically significant), strengthen the findings on the lower binding affinities of branched isomers for HSA based on the calculated Kd's.

Using an equilibrium dialysis method to study the mechanisms of perfluoroalkyl substances (PFASs) molecular interactions with bovine serum albumin (BSA), *Bischel et al. (2010)* reported that, in general, the affinity for albumin increases with PFAS hydrophobicity. Based on the first principles of reverse phase liquid chromatography, which was used in the current study, the more hydrophobic a molecule is, the later it will elute on a reversed phase column. Linear PFOS and PFOA elute later than any of the branched isomers (Figure 4.6), suggesting that the former are more hydrophobic. Thus, our findings that linear PFOS and PFOA are more strongly bound to calf serum are in phase with the findings of *Bischel et al. (2010).*

Although multiple studies have previously established the proteinophillic nature of PFOS and PFOA, which explains in part their long elimination half-lives and their biomagnification potential, this is the first study looking at the isomer-specific binding of PFOA or PFOS, and the

data prove useful for explaining previously observed phenomena. Nevertheless, the current study has some limitations. First, the pure individual linear and branched isomers of PFOS and PFOA are not available for purchase at sufficiently high concentrations from any commercial supplier, and the amounts donated for the current study were completely consumed to produce the data presented in Figures 4.2 and 4.3. Although these data were sufficient for the variant of Scatchard analysis here, we were not able to repeat the study a second time. The strength of the binding to HSA was so strong that working at lower concentrations of isomer (which are commercially available) produced no detectable response in the ultrafiltrates. These facts, in part, explain why there have been no reports of isomer specific protein binding previously, and also explain why there is so little known about the toxicity of individual PFOS and PFOA isomers. The second limitation, compounded by the same reason as above, is that we did not compare the results of the present experiment using the Centrifree® ultrafiltration device with the equilibrium dialysis method, which is still considered as the gold standard method in studies of protein binding.

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CHAPTER 5: Transfer of PFOS and PFOA isomers across an in vitro model of the human placental barrier

Sanjay Beesoon, Joey Sheff, Ashley Davey, Denise Hemmings, Jonathan Martin

Ashley Davey, former graduate student in Hemmings Lab, harvested the cytotrophoblasts from a human placenta, after appropriate ethics clearance from the Health Research Ethics Board of the University of Alberta. Joey Sheff, former undergraduate student in the Martin Lab conducted the biotransformation experiment under the technical supervision of Sanjay Beesoon.

Sanjay Beesoon, designed and conducted the transport assay experiments under the supervision of Denise Hemmings and Jonathan Martin. The manuscript was written by Sanjay Beesoon

Abstract

Perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) are perfluoroalkyl acids widely detected in human blood samples from around the world. These two compounds have been industrially produced for decades and used in a whole array of consumer products and industrial applications. Their traditional manufacturing method until the year 2000 was by electrochemical fluorination (ECF) process which yields a mixture of linear and branched isomers. To date, three biomonitoring studies have shown that the branched isomers are more efficient at crossing the placental barrier compared to the linear molecules.

This phenomenon was surprising given that the linear molecules are more hydrophobic that their branched counterparts. Here we used a monolayer of syncytiotrophoblasts (STs) as an in-vitro model of the placenta to assess the relative transfer efficiencies of linear and branched isomers of PFOS and PFOA. A biotransformation experiment was also conducted with ST cells to evaluate the potential for PFOS precursor metabolism in the placenta, using the model compound N-ethyl perfluorooctane sulfomanide (EtFOSA). Data suggested that STs did not biotransform EtFOSA; however transport assays provided evidence that total PFOA crossed the ST layer more efficiently than total PFOS, and that total branched isomers of PFOS and PFOA crossed the ST monolayer more efficiently than the respective linear molecules. When the data were stratified by individual isomer, the transfer efficiencies of 3*m*- and 4*m*-PFOA were higher than linear PFOA, and 4*m*-PFOS was more efficiently transported across the ST later compared to linear PFOS. This in-vitro model of the placenta may prove useful for the assessment of placental transfer of new chemicals, or emerging contaminants.

5. 1 Introduction

Perfluoroalkyl acids (PFAAs) are a group of emerging environmental toxicants that have been manufactured and used since the 1940's in a wide array of industrial processes and commercial products *(3M Company 1999; Brooke D 2004; Guo Z 2009; Washburn et al. 2005)*. PFAAs are made of a perfluorinated carbon backbone (C_nF_{2n+1} where *n* can range from 4-14) and an acidic functional group, which can be a carboxylic, sulfonic, or phosphonic acid. The two most

commonly studied PFAAs are perfluorooctanoate (PFOA) and perfluorooctanesulfonate (PFOS). In industry, they have been used as coatings on photographic films and papers, as photoresist materials in semiconductors, as acid mist suppressants during metal plating, in hydraulic fluids in the aviation industry, in fire-fighting foams *(Kissa 1994)*. Furthermore, they are used extensively in consumer products such as in surface treatment formulations for carpet, upholstery, apparel, and paper, including for food contact purposes, whereby their main functions are to impart soil, oil, and water repellence *(Buck et al. 2012; Washburn et al. 2005).* The demand for these organic fluorine compounds is related to their resistance to oxidation and thermal stress, resulting from the high carbon-fluorine bond strength, and their surfactant properties of PFAAs at low concentrations. The same physical properties, unfortunately, also impart environmental inertness, including resistance to natural biodegradation processes. As a result of their production, usage over more than 50 years, and their disposition in the environment, these chemicals can now be detected both in the abiotic *(Benskin et al. 2012; Davis et al. 2007)* and biotic environments *(Giesy and Kannan 2001; Kannan et al. 2001)* around the world.

With specific relevance to human exposure, the first documented report on the presence of organic fluorine compounds in human blood was made in 1968 by *Taves et al. (1968).* One compound, PFOA, was later confirmed by gas chromatography in the blood of a few human volunteers by *Belisle and Hagen (1980).* However, it was not until the year 2000 when scientists in the Medical Department of the 3M Company detected four different PFAAs (PFOS, PFOA,

perfluorohexane sulfonate (PFHxS) and perfluorooctane sulfonamide (PFOSA) in archived human serum samples from the general American population. The 3M Company came forward publicly to announce their decision to phase-out their production of "C8 chemistry", including PFOS, PFOA, and related precursor compounds, which until then had been produced by 3M using an electrochemical fluorination (ECF) method which yielded a technical mixture of linear and branched isomers in commercial products *(Reagen 2007)*. The average ratio of linear to total branched isomers in historical ECF production lots were 70%:30% for PFOS and 80%:20% for PFOA *(Reagen 2007*).

These events catalysed a series of environmental fate, toxicology and epidemiological exposure-disease association studies. Among the potential adverse health effects associated with PFAAs, and more specifically to PFOS and PFOA, are neurotoxocity *(Austin et al. 2003; Butenhoff et al. 2009; Mariussen 2012; Sato et al. 2009; Slotkin et al. 2008),* hepatotoxicity *(Benninghoff et al. 2012; Gilliland and Mandel 1996; Hu and Hu 2009; Obraztsov and Shekhtman 1994; Sakr et al. 2007a; Sakr et al. 2007b),* immunotoxicity *(Grandjean et al. 2012; Wang et al. 2011)* disturbance of lipid metabolism *(Eriksen et al. 2013; Nelson et al. 2010; Shankar et al. 2012; Steenland et al. 2010a; Wang et al. 2012),* hyperuricemia *(Geiger et al. 2013; Steenland et al. 2010b),* endocrine disruption *(Knox et al. 2011; Lopez-Espinosa et al. 2011; Spachmo and Arukwe 2012),* low birth weight babies (LBWB) *(Apelberg et al. 2007; Fei et al. 2007; Washino et al. 2009),* breast cancer *(Bonefeld-Jorgensen et al. 2011)* and attention deficit hyperactivity disorder (ADHD) *(Hoffman et al. 2010; Stein et al. 2012*).

With specific relevance to the current work, developmental toxicity associated with PFOS and/or PFOA, has been a major endpoint investigated in multiple rodent studies *(Lau et al. 2003; Thibodeaux et al. 2003; Grasty et al. 2003; Grasty et al. 2005; Abbott et al. 2007a; Abbott et al. 2007b; Abbott et al. 2009; Wolzcf et al. 2007).*

As also discussed in Chapter 2, multiple studies have looked at paired maternal and cord blood concentrations of these emerging toxicants, clearly establishing the transplacental transfer for most perfluoroalkyl substances (PFASs), including PFOS and PFOA *(Beesoon et al. 2011; Fei et al. 2007; Fromme et al. 2010; Hanssen et al. 2010; Inoue et al. 2004; Kim et al. 2011; Midasch et al. 2007; Monroy et al. 2008; Needham et al. 2011; Ode et al. 2013)*. However, only three of these biomonitoring studies *(Beesoon et al. 2011; Gützkow et al. 2012; Hanssen et al. 2010)* have delved into the isomer specific transfer of PFOS and PFOA across the human placental barrier, and all reported higher transplacental transfer efficiencies of the branched isomers (i.e. from electrochemical fluorination) for PFOS. Isomer-specific transfer of PFOA was investigated in only one study (Chapter 2) *(Beesoon et al. 2011*), and the branched PFOA isomers were also found to cross the placenta to a greater extent than linear PFOA – albeit the structure-activity relationship was less pronounced than it was for PFOS. According to Sastry et al. *(Rama Sastry 1999)* the overwhelming majority (> 95 %) of chemical compounds (drugs and xenobiotics) that cross the placental barrier vectorially, from mother to fetus, do so by simple diffusion. Transplacental transfer efficiency (TTE) depends both on the physicochemical properties of the

chemical compound being transferred, and on physiological characteristics of the epithelial cells at the interface of maternal and fetal blood circulation. These specialized multinucleated epithelial cells, known as syncytiotrophoblasts (ST's), cover the placental villi, which are the functional units for chemical exchange between maternal and fetal circulation. The apical side of the cell faces the mother's blood circulation and the basal part is supported by cytotrophoblasts (CT's) which face the fetal blood supply *(Bloxam et al. 1997).* As a matter of fact the ST, which is an extensive fully differentiated multinucleated syncytial layer having an approximate surface area of 12 m^2 at term, is formed by continuous maturation and subsequent fusion of the underlying CT's *(Boyd et al. 1970).* Figure 5.1 below gives an approximate pictorial representation.

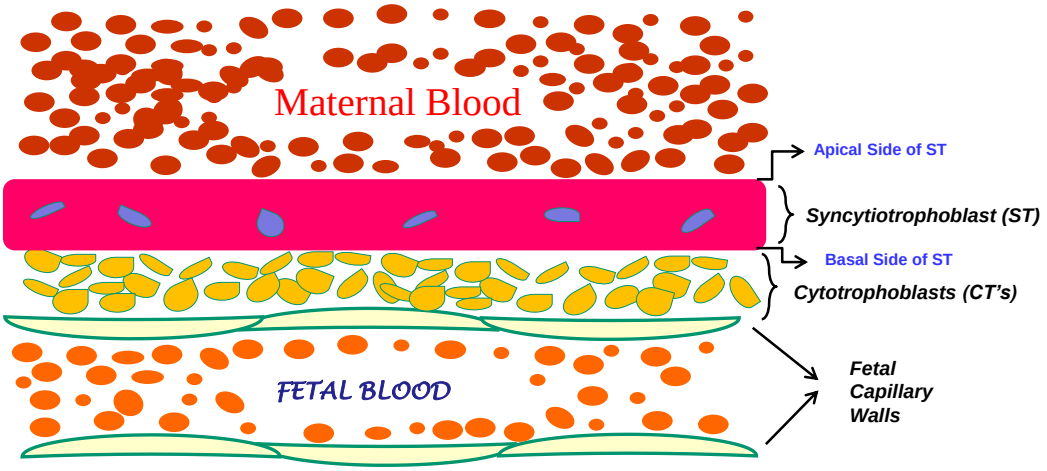

Figure 5.1. Schematic sketch of distribution of CT's and ST's in relation to maternal and fetal blood circulations

The rate of diffusion of any chemical compound is governed by Fick's Second Law of Diffusion, which can be mathematically expressed as:

$$\text{Rate of diffusion} = D \times \Delta C \times A/d$$

where A = area of exchange, d = membrane thickness, ΔC = concentration gradient across the membrane (e.g., $[C_{\text{Maternal Plasma}}] - [C_{\text{Fetal Plasma}}]$) and D = diffusion constant for the chemical of interest. Applying Fick's Law to the current scenario, the only parameter that can potentially explain the different diffusion rates between linear and branched isomers, would be D, the diffusion constant, which is known to be largely dependent on the hydrophobicity of the molecule under investigation *(Hamilton 1998).* Thus, 2 isomeric molecules A and B (same molecular weight), candidates for transplacental transfer, will be discriminated at the placental interface based on their respective hydrophobicities, with the one with a higher hydrophobicity having a higher probability and rate of transfer. Using the above theoretical concept and the fact that the linear molecules of PFOS and PFOA have higher hydrophobicities (e.g. based on the elution order in the reversed phased chromatography) *(Benskin et al. 2007),* TTEs of linear PFOS and PFOA should be higher than those of the branched molecules. The higher TTEs of branched PFOS and PFOA reported in the three above mentioned studies *(Beesoon et al. 2011; Gützkow et al. 2012; Hanssen et al. 2010)* cannot therefore be explained by the simple diffusion model and alternative explanations should therefore be sought. In Chapter 4, data were highly suggestive that preferential binding of linear PFOS and PFOA to maternal or fetal serum proteins may be part of the explanation, but the behaviour of PFAA isomers across the functional unit of the placenta should also be examined.

In addition to simple diffusion across the placental barrier, studies have documented the involvement of active transport proteins in the bidirectional movement of drugs *(Evseenko et al. 2007; Thadani et al. 2004; Unadkat et al. 2004)*, hormones *(Landers et al. 2009; Patel et al. 2011)*, nutrients *(Takahashi et al. 2001)* and toxicants *(Atkinson et al. 2002; Beghin et al. 2010; Gil et al. 2005; Myllynen et al. 2007)* between maternal and fetal circulations. Thus, an alternative explanation for the higher TTEs of branched PFOS and PFOA isomers is that the syncytiotrophoblast cells have the relevant receptors and active or facilitated transport mechanisms that favor the vectorial transport of branched isomers over linear molecules into fetal circulation. To test these hypotheses, we used an in-vitro model proposed by *Bode et al. (2006)* to examine whether branched isomers of PFOS and PFOA can cross a monolayer of syncytiotrophoblasts more efficiently than linear PFOS and PFOA, respectively. To date at least two studies have used this model to test the transplacental transfer of small organic molecules, namely deoxynivalenol *(Nielsen et al. 2011)* and caffeine, benzoic acid, and glyphosate *(Poulsen et al. 2009)*. However, both these former studies used a choriocarcinoma cell line (BeWo) instead of normal human derived placental cells, which we used in the present work.

Although much attention is given to direct PFOS and PFOA exposure, humans are also exposed precursors of these chemicals which have been manufactured in bulk and also used extensively in multiple consumer products *(Paul et al. 2009; Prevedouros et al. 2006)*. Precursor compounds of PFOS, referred to as PreFOS in some scientific literature *(Martin et al. 2010)*, exist in similar technical

mixtures of branched and linear isomers owing to their similar production by the ECF process. Studies on human liver microsomes *(Benskin et al. 2009)* and rodents *(Ross et al. 2012)* indicate that CYP450 and microsomes preferentially metabolize branched PreFOS, which can potentially explain the high branched PFOS isomer content in human and rodent blood *(Martin et al. 2010).* Given the presence of biotransformation enzymes in the human placenta *(Hakkola et al. 1996; Hakkola et al. 1998; Syme et al. 2004),* in particular the cytochrome P450 family, we hypothesized that branched PreFOS may be preferentially metabolized by placental cells, leading to an enhanced branched PFOS isomer composition in fetal circulation, as reported in Chapter 2 from biomonitoring. To test this secondary hypothesis, in the current study we also challenged human placental cytotrophoblasts (CTs) and syncytiotrophoblasts (STs) with a model PreFOS compound, N-ethylperfluorooctanesulfonamide (N-EtFOSA) to determine if placental cells possess the metabolic pathways to biotransform PFOS precursors.

5.2 Materials and Methods

5.2.1 Isolation of Human Placental Cytotrophoblasts (CTs)

Villous cytotrophoblasts were isolated from human placentas as per the method previously described by *Davey et al. (2011).* In brief, chorionic placental tissue was minced, subjected to trypsin/FETase digestion followed by absorption on glass beads coated with immunoglobulins directed at CD9, MHC (Major Histocompatibility Complex) Class I and MHC Class II antigens *(Yui et al. 1994).* The isolated CTs (99.99 % pure) were resuspended in fresh serum-free medium containing 7.5% dimethylsulfoxide. Then, 250 µL of the CT's in

dimethylsulfoxide were aliquoted in polypropylene tubes and cryopreserved in liquid nitrogen until use. For the current study, the CTs were all derived from the same human placenta.

5.2.2 Syncytialization of CTs to STs

Cryopreserved CTs (250 µL) were thawed in a 37° C water-bath and aseptically resuspended in 4.75 mL of Iscove's Modified Dulbecco's Medium (IMDM, purchased from Gibco®, Life Technologies™, Carlsbad, CA, USA) fortified with 10 % fetal bovine serum (FBS, Gibco®) and 1 % penicillin/streptomycin (100 µg/mL each, purchased from Life Technologies™, Carlsbad, CA, USA). This fortified form of IMDM culture medium will be referred to as $IMDM^{++}$. The suspension was gently mixed by inversion and a 15 µL aliquot was taken for a cell count in a hemocytometer. The remaining solution was centrifuged at 1500 rpm for 7 minutes at 4° C (Beckman GS-6R, Beckman Coulter®, Brea, CA, USA). After centrifugation, the cell pellet was resuspended in $IMDM^{++}$ and diluted to a final cell concentration of 6.0×10^5 cells/mL. Then, 200 µL of the cell suspension was dispensed in the 96-well plate (Costar 3595, Corning Inc. Corning, NY, USA) and incubated at 37° C in the presence of 5 % carbon dioxide overnight (Forma Series II 3110 Water Jacketed CO_2 incubator, Thermo Fisher Scientific Inc., Ottawa, ON, Canada). The next day, the 96-well plate was inspected microscopically for adherence of the CTs to the bottom of the wells. The excess cells, and non-adherent cells, were then washed with additional $IMDM^{++}$ and pipetted off. New medium consisting of $IMDM^{++}$ and 0.1 µM cyclic-adenosine monophosphate (8-Bromoadenosine 3′,5′-cyclic monophosphate

(cAMP), purchased from Sigma-Aldrich®, Oakville, ON, Canada) was added to the cell layer and incubated at 37° C overnight. The next day, the cell culture was examined to check whether the CTs had syncytialized to STs, and thus ready for chemical challenge. Syncytialization is essentially the fusion of multiple CT cells to give a multinucleated "giant" cell, the ST, which in a human placenta represent the apical interface between maternal and fetal blood circulation. This is an overly simplified description of syncytialization, which is a far more complex process connecting a whole spectrum of programmed cellular events and involving many proteins *(Huppertz 2006; Toufaily et al. 2013; Pidoux et al. 2012)*. For the isomer–specific transport assays, CTs were cultured on Nunc® cell inserts (Thermo Scientific) having a culture area of 0.47 cm^2, a pore size of 0.4 µm, and pore density of $< 0.85 \times 10^8$ pores/cm^2 in each well. Prior to use, the cell culture surfaces of the inserts were coated for 1 hour with human collagen (purchased from Sigma Aldrich) at a concentration of 10 µg/cm^2 and then washed with sterile phosphate buffered saline to remove excess coating solution. The same cell culture protocol as above was used to seed the CTs onto the inserts and their subsequent treatment with cAMP to produce STs.

5.2.3 Confirmation of ST viability and formation

$IMDM^{++}$ overlaying the ST cell layer was sampled prior to the chemical challenge and kept at -20° C pending analysis for β-HCG (Human Chorionic Gonadotropin) hormone, which is a critical element for the maintenance and progress of pregnancy *(Cole 2012)*. β-HCG analysis was performed by an enzyme linked immune sorbent assay (ELISA) method, using a kit purchased from DRG

diagnostics (Marburg, Germany). Briefly, 25 µL of each standard solution, negative controls (distilled H_2O, plain $IMDM^{++}$) and samples (IMDM in contact with STs) were added to microtiter wells followed by 100 µL of the enzyme conjugate (anti β-HCG antibody conjugated to horseradish peroxidase) and wells were incubated at room temperature (25° C) for 1 hour. Wells were rinsed 5 times with 80 µL water each time and 100 µL of substrate solution (tetramethylbenzidine) was added to each well. The microplate was incubated for another 15 minutes at room temperature and the reaction was stopped by adding 50 µL of stop solution (0.5 M H_2SO_4) to each well. Absorbance (optical density) was measured at 450 nm (within 10 minutes of adding stop solution) with an EL 808 absorbance microplate reader (Bio-Tek Instruments Inc., Winooski, VT, United States). Whenever β-HCG concentrations fell outside of linear dynamic range of the calibration curve, the relevant samples was diluted (1 in 10 or 1 in 100) with sample diluent. β-HCG was consistently detected in the culture media with concentrations ranging from 180 mlU/mL to 1124 mlU/mL.

Confluence of the ST layer on the inserts (for the transport assays) was assessed under the microscope throughout the duration of the experiment. After the experiments were completed, the integrity of the ST monolayer was confirmed by hematoxylin stain and visualization under the microscope.

5.2.4 PreFOS biotransformation experiment

Prior to challenging cells, medium was removed and 200 µL methanol was added to 4 wells (from a total of 20) to kill cells to be used as a negative controls. Wells were washed (2 x 100 µL) with phosphate buffered saline. Medium was then

removed from all wells, and replaced with 4 µL of stock solutions of EtFOSA in 200 µL IMDM++. STs were incubated for 24 h at 37° C after which medium was collected. Bottom of wells were scraped and washed with 2 x 150 µL MeOH to ensure collection of all content. Medium was stored in amber HPLC vials at -20° C for PFAA analysis. For each assay, a single row of cells was killed by removing medium, and adding MeOH, and once again replacing MeOH with new medium prior to chemical challenge. If the STs have the biotransformation capacity to metabolize EtFOSA, then FOSA would be formed as the metabolite with a simultaneous EtFOSA depletion; as demonstrated by *Benskin et al. (2009)* with cytochrome P450 and liver microsomes. FOSA and EtFOSA concentrations were both monitored by HPLC-MS/MS.

5.2.5 Transport Assays

Br-PFOS and T-PFOA standards, containing a technical mixture of linear and branched isomers of PFOS and PFOA, respectively, were diluted in plain IMDM (free from fetal bovine serum) to reach final concentrations of 10 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL. To conduct the transport assays, a 24-well cell culture plate was used. 300 µL of each dilution was added to inserts containing the ST cell layer, representing the maternal side and 900 µL of plain medium was added to the wells, representing the fetal side (Figure 5.2). To examine whether PFOS or PFOA isomers bound to the inserts, parallel control experiments were run using inserts without the ST cell layer to ascertain whether the collagen coating on the inserts, or the polycarbonate material of the insert itself, may bias the result of a possible differential transport of branched and linear isomers

through selective adsorption. 20 µL aliquots of medium in the insert, and in the well (fetal side), were sampled at 1 hour and 2 hours and stored in Eppendorf tubes at -20° C pending analysis by HPLC MS/MS.

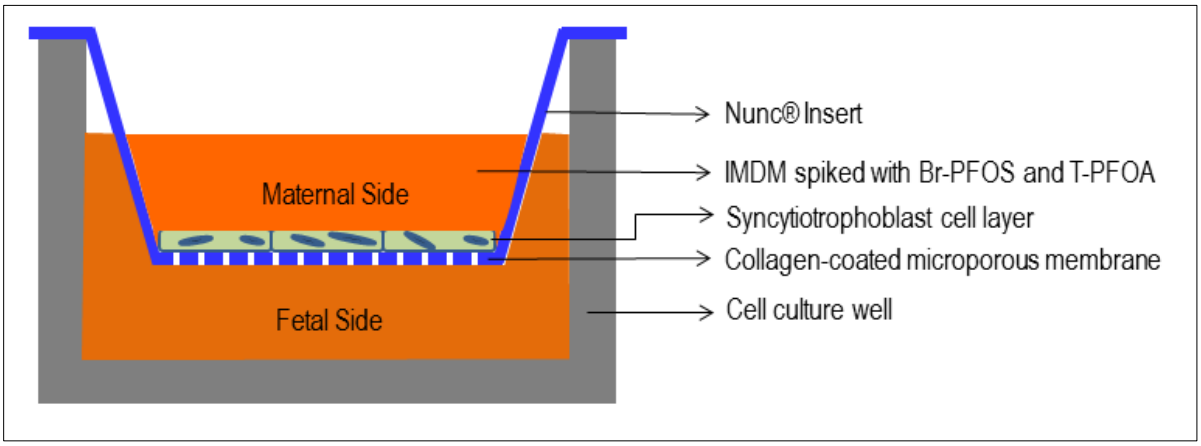

Figure 5.2. Cross-sectional schematic view of a cell culture well with the insert and ST cells dividing the maternal and fetal compartments.

5.2.6 Liquid Chromatography-Tandem Mass Spectrometry

Samples from both the transport assay and the biotransformation experiment were analysed by the same HPLC-MS/MS method, but the sample prepartion varied slightly. For the transport assay, 20 µL samples were spiked with 5 ng (10 µL) of mass labelled internal standards (MPFAC-MXA purchased from Wellington Laboratories, Guelph, ON, Canada) and were further diluted to 150 µL with HPLC grade methanol. Contents of the tubes were thoroughly mixed on a vortex and centrifuged at 14,000 rpm. Then, 100 µL of the supernatants were transferred to HPLC vials for analysis. For the biotransformation experiments, samples were spiked with 5 ng of the mass labelled internal standard, mixed on a vortex, centrifuged at 14 000 rpm, and 100 µL was sampled for HPLC-MS/MS analysis.

The isomer specific method of *Benskin et al. (2007)* was adapted for the chromatographic separation of the PFAAs on a FluoroSep-RP Octyl HPLC column (3µ 100Å, 15cm x 2.1mm column, ES Industries, West Berlin, NJ, USA) and their detection by MS/MS. Samples from the transport assays were analyzed using a Shimadzu UFLC-XR (Kyoto, Japan) coupled to a QTrap 4000 mass spectrometer (MDS Sciex, Concord, ON, Canada). Samples for the biotransformation experiment were run on an Agilent 1100 series LC system and mass spectral data were collected on an API 5000Q mass spectrometer (MDS Sciex, Concord, ON, Canada). The same HPLC starting conditions were used in both cases. Flow rate was 150 µL/min, and starting conditions were 60% A (water adjusted to pH 4.0 with ammonium formate) and 40% B (methanol). This was maintained for 0.3 min, followed by subsequent increments of mobile phase B to 64% by 1.9 min, 66% by 5.9 min, 70% by 7.9 min, 78% by 40 min, and 100% by 42 min, and held there until 65 min. Multiple reaction monitoring (MRM) was used to collect data in electrospray negative-ion mode. Peaks were integrated manually with Analyst 1.5 Software (MDS Sciex, Concord, ON, Canada). To confirm the identity of a particular compound, at least 2 ions were monitored for that compound, but only the most sensitive ion, free from interference, was used for quantitative purposes.

5.3 Results and Discussion

5.3.1 Biotransformation of EtFOSA

While a tenfold drop of EtFOSA levels was seen at 10 ng/mL, 20 ng/mL and 50 ng/mL spiking concentrations, we observed no significant corresponding increase

in FOSA (the P450 metabolite) concentration above blank readings (Figure 5.3). These very low levels of FOSA only permitted detection of the linear isomer, thus we could not confirm formation of branched FOSA isomers.

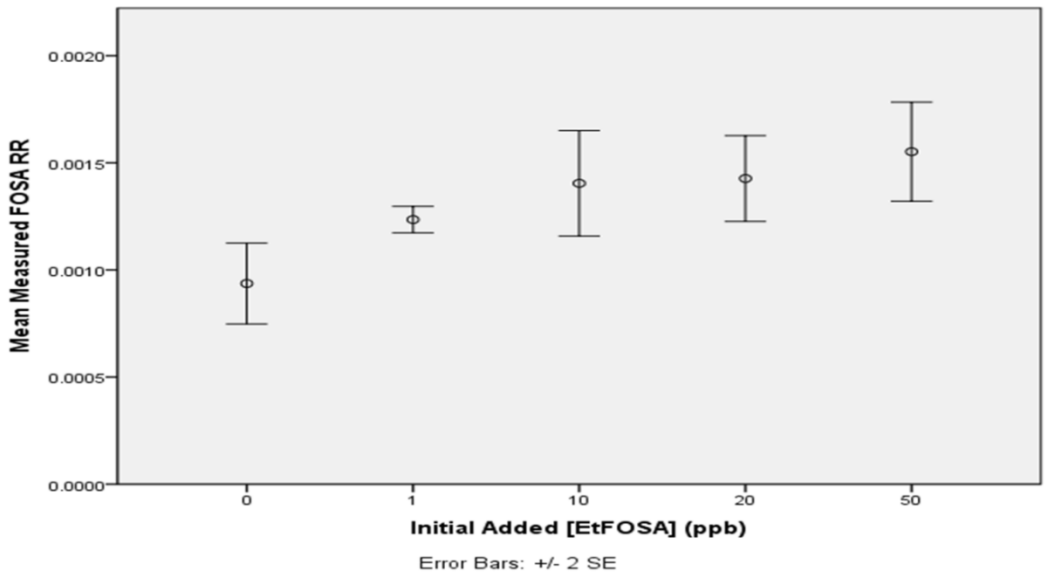

Figure 5.3. Mean relative response of FOSA metabolite (relative to 1ppb mass-labeled FOSA) at control and initial [EtFOSA] in incubation medium. No statistically significant increase in FOSA was observed with increasing dose.

These experiments were done in triplicate and were repeated on three different days. To examine if EtFOSA volatiled out of the culture, aided by air circulation in the incubator, the experiments were repeated using parafilm to seal the cover to the microtitre plate, but the same results were found. Two possible explanations are thus offered for the drop in EtFOSA (substrate) without any significant production of FOSA (product). First, the EtFOSA spiked in the medium was readily adsorbed onto the surface of the cell culture plate (made of polycarbonate plastic) and thus was unavailable to the STs to be biotransformed to FOSA. The second possibility is that EtFOSA may well have been biotransformed by the STs, but to a compound other than FOSA *(Benskin et al. 2009b)*, such as a Phase II

conjugate, but no standards were available and these were not monitored. The results from this work were inconclusive.

5.3.2 Transport Assays

5.3.2.1 Total PFOS and PFOA Transport

The mean transfer efficiencies ± 95% C.I of total PFOA and PFOS (i.e. the sum of all branched and linear isomers) at two time points, and at four different concentrations, are shown in Figures 5.4 and 5.5, respectively. Using PFOS as an example, the following mathematical equation was used to calculate transfer efficiency, where RR represents the relative response of the peak area with respect to the peak area of mass-labelled linear PFOS:

$$Transfer\ Efficiency = \frac{RR_{PFOS}^{Basal}}{RR_{PFOS}^{Apical} + RR_{PFOS}^{Basal}}$$

Analysis of the whole dataset by 3-way ANOVA showed that the transfer of total PFOA was greater than total PFOS ($p<0.001$), that transfer at 2 hrs was greater than at 1 hr ($p<0.001$), and that there was furthermore a significant concentration effect ($p<0.001$). The result here, whereby PFOA crossed the in vitro placental barrier to a greater extent than PFOS, is consistent with the biomonitoring described in Chapter 2, and also with many literature reports of placental transfer for PFOS and PFOA *(Fei et al. 2007; Fromme et al. 2010; Hanssen et al. 2010; Inoue et al. 2004; Kim et al. 2011; Midasch et al. 2007; Monroy et al. 2008; Needham et al. 2011).*

To further understand the concentration and time effects, described above from 3-way ANOVA, PFOA and PFOS datasets were each then analyzed separately by 2-way ANOVA. For PFOA, 2-way ANOVA also indicated that transfer of total PFOA increased significantly between 1 and 2 hours ($p<0.001$) and furthermore confirmed a concentration ($p<0.001$) effect. Post-hoc Bonferroni multiple comparisons were then made, and this showed that the transfer of PFOA was significantly greater at 10 ppb than at all higher concentrations ($p<0.001$ for all 3 comparisons), but that there was no significant difference among transfer efficiencies for 50, 100, and 200 ppb. For PFOS, 2-way ANOVA indicated no significant effect of concentration, but did indicate a statistically significant increase of the transfer efficiency between 1 and 2 hours ($p= 0.016$).

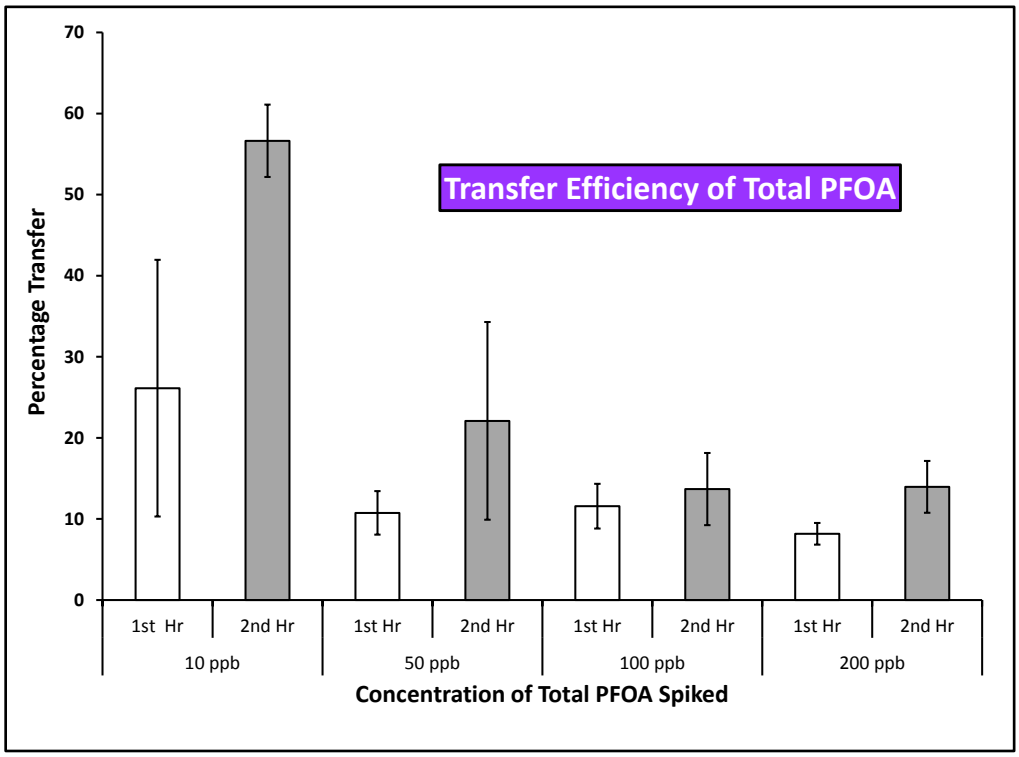

Figure 5.4. Transfer efficiencies of total (linear and branched) PFOA across the in vitro syncytiotrophoblast layer. Error bars represent 95% confidence interval.

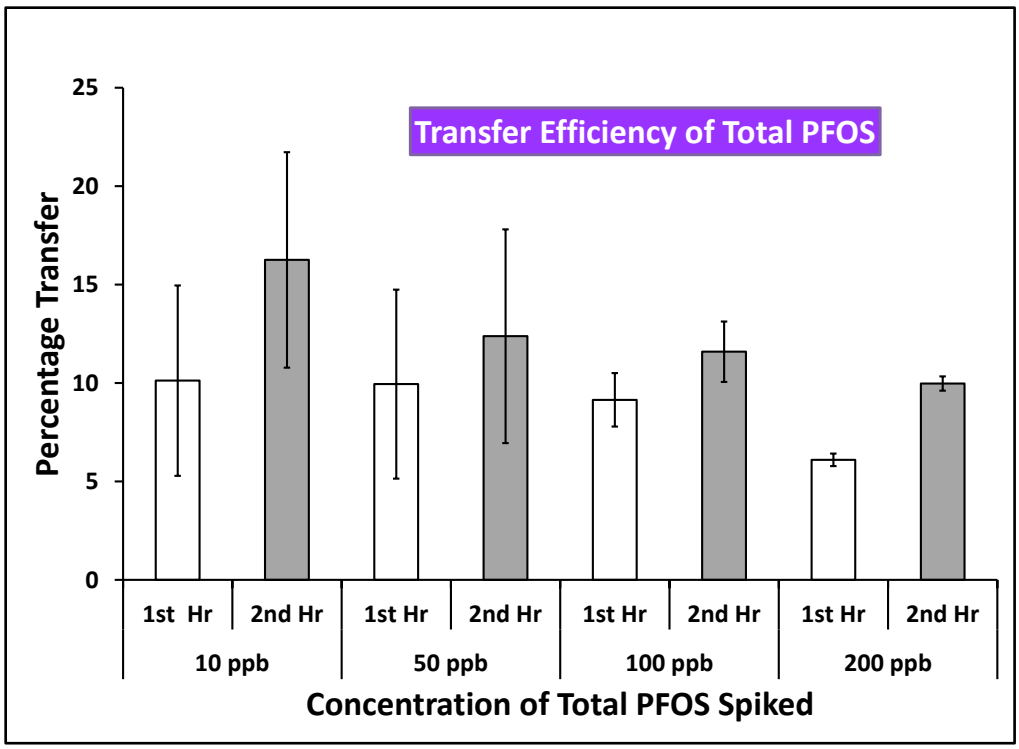

Figure 5.5. Transfer efficiencies of total (linear and branched) PFOS across the in vitro syncytiotrophoblast layer. Error bars represent 95% confidence interval.

5.3.2.2 Isomer-Specific PFOS and PFOA Transport

To consider the relative efficiencies of linear and branched isomers at crossing the syncytiotropoblast monolayer, the percentage of total branched isomers (%Br) of PFOS and PFOA in the maternal and fetal side of the cell culture system was calculated. Then, the difference (% Br maternal - % Br fetal) between the two chambers was calculated for each time and concentration (Figure 5.6 for PFOA, Figure 5.7 for PFOS). As shown in both Figures 5.6 and 5.7, most data showed a tendency for enrichment of branched isomers on the fetal side of the cell culture, shown by a downward vertical bar in these charts. Where the 95% confidence intervals do not cross zero in these plots, this demonstrates statistically significant enrichment of branched PFOA in the fetal side.

For PFOA, significant enrichment of the branched isomers on the fetal side was shown at 2 hours for 10 ppb, 50 ppb, and 200 ppb spiked samples. Two-way ANOVA indicated that enrichment of branched isomers on the fetal side increased significantly between 1 and 2 hours ($p=0.025$), and furthermore indicated a significant concentration effect ($p=0.005$). Post-hoc Bonferroni multiple comparisons were then made for PFOA, and this demonstrated that at 10 ppb the enrichment of branched isomers on the fetal side was significantly greater compared to at all three of the higher spiked concentrations (when comparing 10 ppb to 50, 100, and 200 ppb, *p*-values were 0.048, 0.01, and 0.013, respectively).

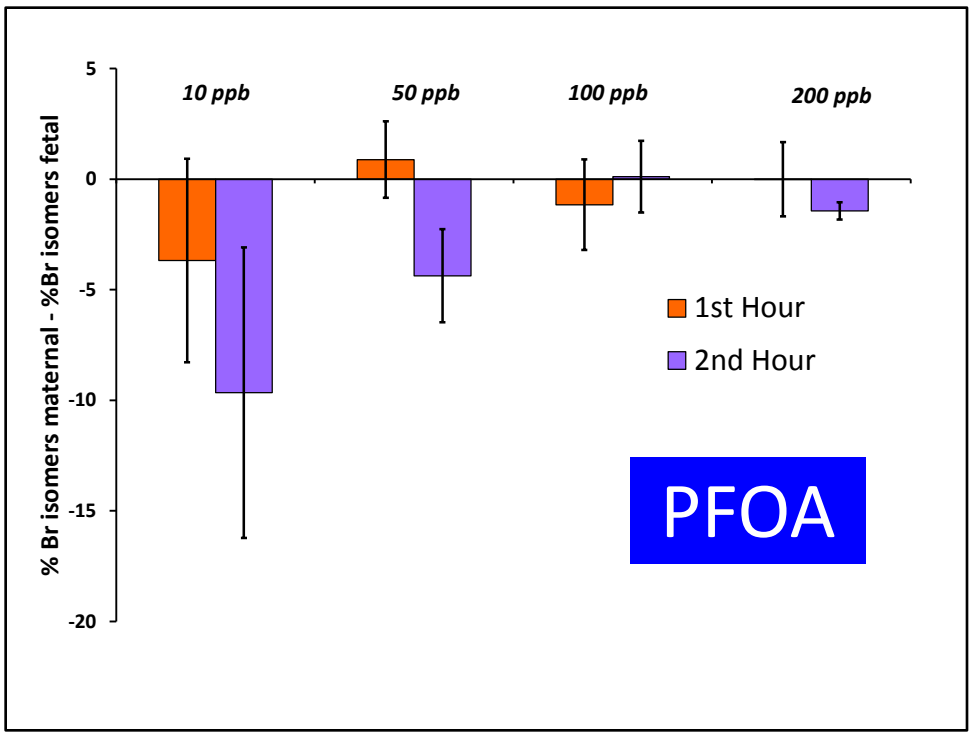

Figure 5.6. Transfer efficiencies of total branched PFOA across syncytiotrophoblast layer. A negative bar indicates higher branch isomers in the fetal side. Error bars indicate 95% confidence intervals. Whenever the error bars cross the zero axis, it is indicative of a non-statistically significant difference at the 95% confidence interval.

For PFOS, statistically significant enrichment of the branched isomers on the fetal side was only shown at 10 ppb (at both 1 and 2 hrs.), and at 100 ppb (only at 2 hrs.). Two-way ANOVA for PFOS indicated no significant effect (p=0.19) of time (1 or 2 hours), but did indicate a significant concentration effect (p=0.01). Post-hoc Bonferroni multiple comparisons were then made for PFOS, and this demonstrated that at 10 ppb the enrichment of branched isomers on the fetal side was significantly greater compared to 100 ppb (p=0.03) and 200 ppb (p=0.03). It is clear from Figure 5.7 that the effect did indeed diminish at higher concentration.

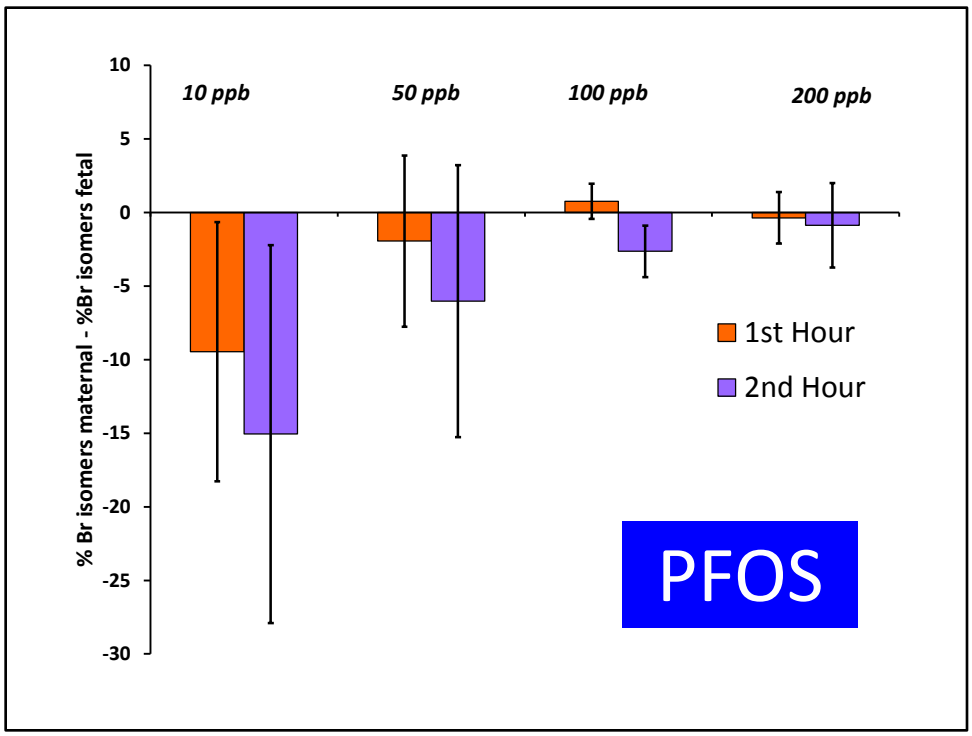

Figure 5.7. Transfer efficiencies of total branched PFOS across Syncytiotrophoblast layer. A negative bar indicates higher branch isomers in the fetal side. Error bars indicate 95 % confidence interval. Whenever the error bar crosses the zero axis, it is indicative of a non-statistically significant difference at 95% confidence interval.

To examine for any isomer-specific effect, the transfer efficiencies of the individual branched PFOS and PFOA isomers were indexed to the transfer efficiency of their respective linear molecule, which we termed relative transfer efficiency (RTE). Given that some of the individual branched isomers were not above detection limits in the fetal side after the first hour of the experiment, or at either time in the 10 ppb experiments, only data from the second hour were used for these calculations, and only at 50 ppb, 100 ppb and 200 ppb. These data were pooled together to give more power to the analysis.

Using 1*m*-PFOS as an example, the following equation was used to calculate its RTE, whereby RR represents the relative response of the peak area with respect to the peak area of mass-labelled internal standard.

$$RTE\ of\ 1mPFOS = \frac{\left[RR^{Basal}_{1m\ PFOS} \Big/ RR^{Basal}_{Linear\ PFOS}\right]}{\left[RR^{Apical}_{1m\ PFOS} \Big/ RR^{Apical}_{Linear\ PFOS}\right]}$$

Thus, RTE's greater than one indicate a higher transfer efficiency for a particular branched isomer, compared to the linear isomer, and RTE's lower than one indicate the opposite.

For PFOS, box-plots of RTE for the individual branched isomers are shown in Figure 5.8. These results indicate that the only isomer of PFOS which crosses the ST layer more efficiently than linear PFOS is 4*m*-PFOS (p<0.05 when tested if RTE was significantly different from 1.0). The structure-activity relationship reported for PFOS in Chapter 2 of this thesis, whereby the closer the perfluoromethyl branching point was to the sulfonate group the higher was the TTE, was not visible here.

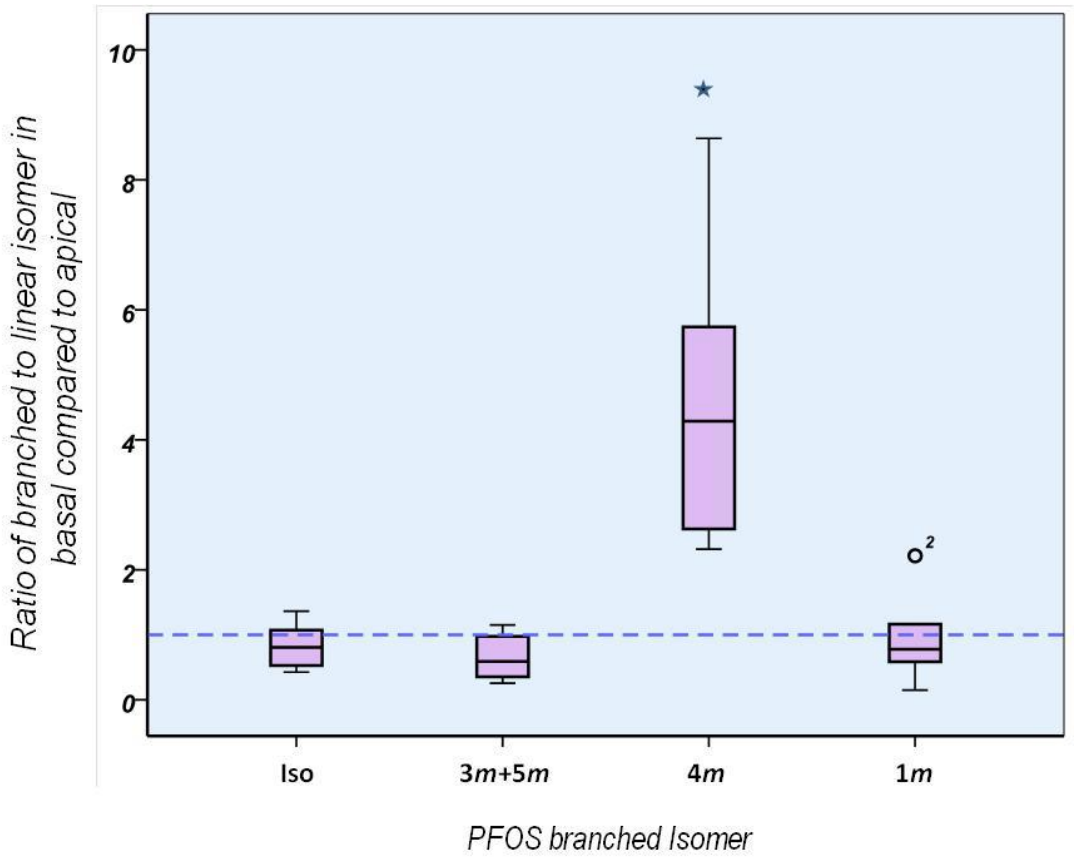

Figure 5.8. Relative transfer efficiencies of individual branched PFOS isomers across ST layer. Empty circles above the box-whisker plots represent outliers. The asterisks above the whiskers indicate statistically significant differences of the means RTEs from 1.

For PFOA, the mean RTEs of iso-, 5*m*- and 4*m*-PFOA were statistically significantly different from 1.0 (Figure 5.9), indicating that these branched PFOA isomers were more efficient at crossing the ST layer compared to linear PFOA. For 3*m*-PFOA, the difference with respect to linear PFOA was only marginally statistically non-significant at the *95% CI*, as the lower bound (0.97) was very close to 1.0. These data confirm findings reported in Chapter 2 of the thesis to the effect that branched PFOA isomers do indeed cross the human placental barrier more efficiently that linear PFOA, but that there is no strong structure-activity among the branched isomers.

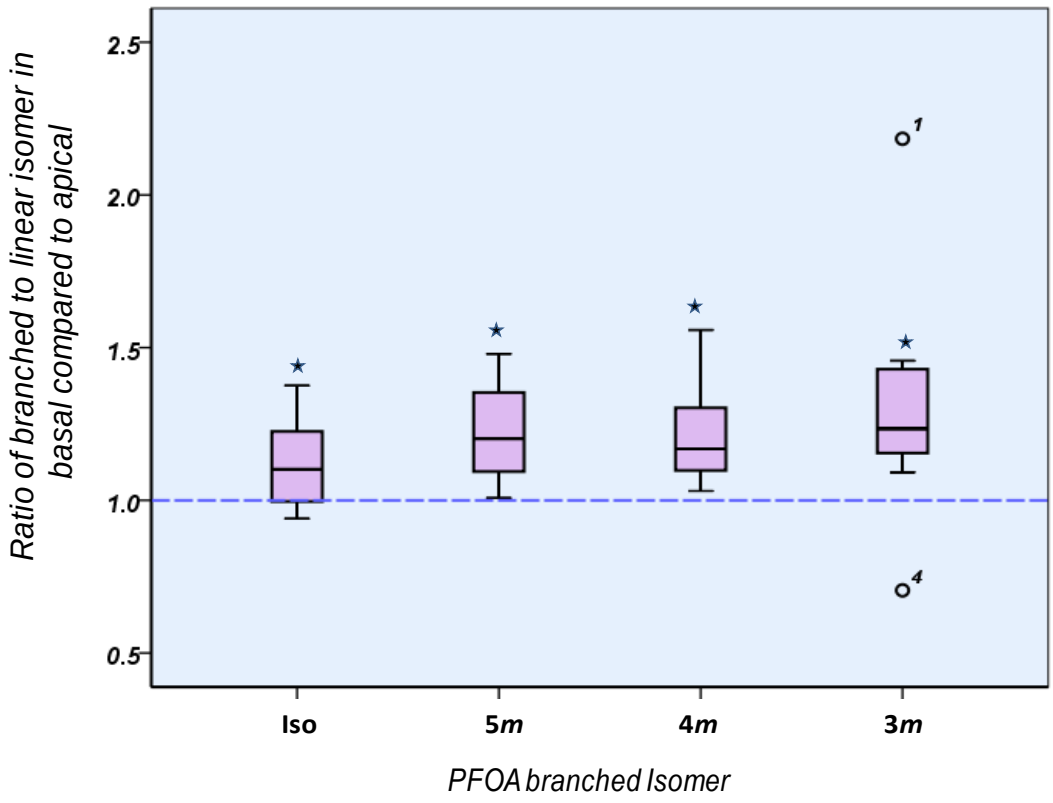

Figure 5.9. Relative transfer efficiencies of individual branched PFOA isomers across ST layer. Empty circles above and below the box-whisker plots represent outliers. The asterisks above the whiskers indicate statistically significant differences of the means RTEs from 1.

5.3.3 General Conclusion

Two important findings shown in the current study confirm trends that are seen from biomonitoring of paired maternal and fetal sera. Firstly, the higher transfer efficiency of total PFOA compared to total PFOS across the ST layer validates earlier findings based on the trace analysis of paired maternal and fetal serum samples, whereby the ratio of cord to maternal concentrations range from 0.61 to 1.26 for PFOA, but only from 0.30 to 0.60 for PFOS. Secondly, the higher transfer efficiencies of branched isomers of PFOS and PFOA compared to the linear molecules found in this in-vitro experiment are in line with isomer specific analysis of paired maternal and cord serum samples *(Beesoon et al. 2011; Gützkow et al. 2012; Hanssen et al. 2010).*

Chapter 4 of this thesis pointed to one of the possible reasons why isomers of PFOS and PFOA cross the placental barrier more effectively than the linear molecules. Specifically, the binding affinities of linear PFOS and PFOA were stronger for human serum albumin, and total serum protein, compared to branched isomers; leaving relatively enriched proportions of unbound branched isomers of PFOS and PFOA to diffuse, or be transported, across the placental ST layer. In the current study the culture media used during the transport assays were free of any protein, thus the current mechanism of preferential branched isomer transfer would serve only to enhance the relative preferential transfer of branched PFOS or PFOA isomers across the placenta in vivo.

Putting together the facts that, first, branched and linear isomers have the same molecular weights; second, that linear molecules have higher hydrophobicities

than branched isomers; and third, that the transfer efficiencies of branched isomers are higher than linear in the absence of albumin, a logical explanation for the current findings is that the STs have an active or facilitated transport mechanism that preferentially transfers branched isomers from the maternal to the fetal circulation. The findings here that the percentage transfer of total PFOA and PFOS decreased with increasing concentrations (Figures 5.4 and 5.5 respectively) is also suggestive of a protein dependent transport mechanism, which would begin to saturate at high concentrations of substrate.

The preferential transport of branched PFOS and PFOA isomers over the linear molecules is not an isolated finding. There are data in the scientific literature documenting that the transfer efficiencies of branched amino acids are higher than the linear ones *(Carroll and Young 1983, Christensen 1979)* and furthermore that for some amino acids placental transport is stereospecific *(Eaton et al. 1982).*

Overall, data from the current study are consistent with the biomonitoring of paired maternal and fetal samples showing higher TTEs of total branched isomers of both PFOS and PFOA, although in chapter 2 a structure-activity relationship was found for PFOS, but not for PFOA.

This in-vitro model of the human placental barrier, using normal human placental cells, can be effectively used to make a quick assessment of the potential of other xenobiotics to cross from mother to baby. However, this technique needs further validation with model compounds, before its full potential can be exploited in risk assessment for potential developmental toxicants.

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CHAPTER 6: Summary, Conclusions and Future Work

This thesis is the fruit of a multidisciplinary approach, combining both fundamental laboratory–based and public health research methodologies, to investigate the manufacturing source of PFAAs in human exposure media, pathways of human exposure, and the disposition of perfluoroalkyl acids after they are in our blood.

With the phase-out of electrochemical (ECF) production of PFOS and PFOA by the 3M Company in 2002, and a ramping up of PFOA production by telomerization by several other companies soon thereafter, a big question that needed to be answered was whether current human exposure was/is to historical 3M Company PFOA, or contemporary (e.g. Dupont and other companies) PFOA produced by telomerization. Using a sophisticated isomer-specific analytical method to analyze house dust it was possible to show we are currently exposed to both ECF and telomer PFOA. It would be interesting to repeat surveys such as this one in other populations (including in other countries), in a prospective manner (repeated samples over several years going forward), and in other environmental exposure media such as drinking water and food, not just dust.

With previous knowledge on the transmission of PFAAs from mother to fetus, and a growing body of evidence on the developmental toxicity of PFAAs, another important question was whether the fetus is exposed to the same isomer signature of PFAAs as the mother, and whether the maternal-fetal transfer is chain length

dependent. In Chapter 2, analysis of 20 paired maternal and cord blood samples was conducted, and to our surprise the branched isomers of PFOS and PFOA were more efficient at crossing the human placenta compared to the linear molecules, and a strong structure-activity relationship was furthermore noted for PFOS. Consistent with the results for PFOS isomers – that the *more hydrophilic* branched isomers crossed the placenta to a greater extent that the *more hydrophobic* linear isomer - longer chain PFAAs were less efficiently transported across the placenta than shorter ones, and total PFOS crossed to a lesser extent than total PFOA. These data led us to two hypotheses, which were tested in Chapters 4 and 5.

Firstly, the binding affinity of branched PFOS and PFOA isomers to human serum albumin was compared with the linear molecules in Chapter 4. A model used in pharmaceutical sciences to estimate the fraction of free drugs in plasma was adapted to calculate the dissociation constants of three individual branched and linear PFOS and PFOA isomers. The findings on the lower dissociation constants (thus, higher binding affinities) of the linear molecules gave a logical explanation, not only to the higher transplacental transfer efficiencies of branched isomers, but also to the higher renal clearance elimination efficiencies of branched isomers – a study I co-authored with Dr Yifeng Zhang *(Zhang et al. 2013).* The peculiar results for 1*m*-PFOS, that it bound most strongly to total human serum proteins compared to all other isomers, and that this result correlates with the results of *Zhang et al (2013)*, whereby 1*m*-PFOS was the most persistent PFAA in humans (half-life 90 years), provides compelling evidence that strong protein-

binding of PFAAs is likely the major factor controlling the elimination half-life for humans.

Secondly, a cell-culture model was developed on inserts, mimicking the maternal-fetal interface of the placenta. Using this system, without any protein introduced in the cell culture medium, thus making linear and branched isomers equally bioavailable to cross a monolayer of syncytiotrophoblasts, it was found that the branched molecules were transported more effectively from the maternal side to the fetal side. This finding, along with a concentration dependence, suggested the presence of transporter proteins in the human syncytiotrophoblasts that could preferentially transport branched PFOS and PFOA isomers into fetal circulation.

With the 3M decision to stop PFOS manufacture in 2002, it was not immediately clear what substitutes they had decided to use in their contemporary line of Scotchgard™ carpet protector products. The findings of exceptionally high serum PFHxS concentrations in 2 members of the same family by our colleague, Dr Genuis, expedited a full-fledged investigation on the sources and pathways of exposure in the associated family (seven individuals). Analysis of serum, urine and feces samples from the family members as well as carpet, dust and air samples in their house led to the conclusion that the source of contamination was the house carpet, which was treated several times with Scotchgard in the preceding years, and that the pathway of exposure was most likely through inhalation and ingestion of contaminated house dust. A thorough probing of 3M documents and other published literature revealed that at the time of the PFOS

phase-out, PFHxS seemed to have been used as a short-term substitute in 3M Scotchgard™ carpet protector products.

However, given the longer elimination half-life of PFHxS (8.5 years) compared to PFOS (≈ 4.5 years), and the fact that recent epidemiology *(Hoffman et al. 2010; Gump et al. 2011; Stein and Savitz 2011; Gallo et al. 2013)* and toxicology *(Cassone et al. 2012; Viberg et al. 2013; Lee and Viberg 2013)* studies are pointing to PFHxS as a potential risk to human health, the move of the 3M Company to replace PFOS by PFHxS in the Scotchgard™ formulations around the year 2000 was not a good one, in hindsight.

Overall, this thesis made some key findings in the sphere of human exposure assessment of PFAAs, particularly in the field of isomer-specific pharmacokinetics and provides logical explanations for their behavior at the level of the placenta and kidneys. It is anticipated that this work will open new research avenues on the isomer specific toxicities of these emerging environmental toxicants. The work on isomer specific transfer calls for research on the presence of receptors and transporters in human placental cells, not only for PFAAs but for xenobiotics in general. The need for further research in the role of the placenta to either protect the fetus against xenobiotics, or bioactivation of toxicants through the placental biochemical machinery was also raised by *Prouillac and Lecoeur (2010).* This is especially important given the increasing concern of public health authorities over adverse developmental outcomes of environmental toxicants such as, for example, bisphenol-A (BPA) and its metabolites. Last but not least, the findings on the family study (Chapter 2) highlight how little we know about how

PFAAs were used, and how human exposure occurs (including social determinants), particularly for subpopulations receiving very high exposures. Discovery of new exposure pathways, or new PFAAs or their precursors in environmental or human samples are still likely to come to light.

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