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Prenatal Exposure to Perfluoroalkyl Substances and the Risk of Autism, Attention Deficit Hyperactivity Disorder and Cerebral Palsy in Children - A Danish National Birth Cohort Study

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A dissertation submitted in partial satisfaction of the requirements

for the degree

Doctor of Philosophy in Epidemiology

by

Ze Yan Liew

2014

ABSTRACT OF THE DISSERTATION

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Autism, Attention Deficit Hyperactivity Disorder and Cerebral Palsy in Children
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By

Ze Yan Liew

Doctor of Philosophy in Epidemiology

University of California, Los Angeles, 2014

Professor Beate Ritz, Chair

Perfluoroalkyl Substances (PFASs) are a group of man-made fluorine-containing compounds that are used broadly in industry and commercial products since the 1950s. PFASs are very persistent in the environment and in living organisms, and thus are detected in wildlife and in humans throughout the globe. PFASs can cross the placental barrier and expose the fetus during the most vulnerable period in development. Animal data suggested that PFASs are neurotoxic and have endocrine disruptive properties. A few cross-sectional studies linked current level of PFASs to hyperactivity disorders in children, but finding from prospective studies are lacking. Additional human studies are urgently needed to examine the potential neuro-developmental impacts of these ubiquitous exposures.

We conducted a case-cohort study within the framework of the Danish National Birth Cohort (DNBC) to investigate whether prenatal exposure to PFASs increases the risk of infantile autism, attention-deficit/hyperactivity disorder (ADHD) or congenital cerebral palsy (CP) in children. Among 83,389 mother-child pairs enrolled in the DNBC during 1996–2002, we identified 890 ADHD cases and 301 autism cases in the Danish National Hospital Register and the Danish Psychiatric Central Registry. We also identified 156 CP cases from the Danish National Cerebral Palsy Register. For cost-efficiency, we randomly selected 220 cases of ADHD and autism each, and all 156 CP cases in the three disease groups. In addition, we randomly selected 550 controls from the cohort frequency matched on child's sex. Sixteen` PFASs were measured in maternal plasma samples from early or mid-pregnancy, but in analyses we focus on the six PFASs that were quantifiable in more than 90% of the samples. PFAS concentrations were analyzed as continuous variables (with natural-log transformation) or categorized into quartiles. We estimated Odds Ratios (OR) and Risks Ratios (RR) for the outcomes.

First, we found that prenatal exposures to PFASs may increase the risk for CP in boys. We observed higher risks of CP in boys with increasing maternal PFAS levels (RR=1.74 (95%CI 1.05-2.88) per one unit (natural-log ng/mL) increase in perfluorooctane sulfonate (PFOS) and RR=1.99 (95%CI 1.15-3.44) per unit increase in perfluorooctanoic acid (PFOA)). We also observed a dose-response pattern of CP risks in boys per PFOS and PFOA quartile (p-trend<0.01). Both spastic unilateral or bilateral CP sub-phenotypes were found to be associated with PFASs, but no association between PFASs and CP was found in girls. For ADHD and autism, we found no consistent patterns to suggest that prenatal PFAS exposures increase the risks of ADHD or autism in children, but some observed positive as well as inverse association in secondary analyses should be further explored.

We also conducted a bias analysis to investigate whether conditioning on live-born status may induce sufficiently large bias to explain some of the unexpected inverse associations seen between prenatal PFAS exposures and ADHD in our and previous studies. We employed directed acyclic graphs to present our causal assumptions and the hypothesized scenarios, and used Monte-Carlo techniques to simulate a realistic pregnancy cohort based on the distributions in the DNBC and prior research knowledge. We found that the protective associations of PFAS on ADHD observed in the live-born cohort could possibly be explained by selection bias if PFAS reduces fetal survivals, while PFAS has a true null effect on ADHD. The magnitude of this selection bias due to fetal death are generally small, unless the exposure is a strong determinant of fetal loss and there are one or more strong risk factors of the outcome that also reduce chances of fetal survival. One way to reduce or even eliminate the bias due to conditioning on fetal survival is to adjust for the common causes of the outcome and fetal losses in analyses.

In conclusion, we found that prenatal exposure to PFASs may increases the risks for CP in boys, and no consistent associations were found between maternal PFAS levels and ADHD or autism in children. In additional bias analysis, we quantitatively showed that conditioning on life-born status in observational study can yield a negative bias if an exposure of interests reduces conception or fetal survival among the high risks fetuses for the targeted outcome.

The dissertation of Ze Yan Liew is approved.

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2014

DEDICATION

I dedicate my dissertation work to my beloved family, my dearest teachers and friends.

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ABBREVIATIONS

Perfluoroalkyl Substances (PFASs)

Perfluorinated chemicals (PFCs)

Congenital cerebral palsy (CP)

Danish National Birth Cohort (DNBC)

Danish National Cerebral Palsy Register (DNCPR)

Risk Ratios (RR)

95% confidence intervals (CI)

Lower limit of quantitation (LLOQ)

Odds Ratios (OR)

Perfluorooctanoic acid (PFOA)

Perfluorooctane sulfonate (PFOS)

Perfluorohexane sulfonate (PFHxS)

Perfluorononanoic acid (PFNA)

Perfluoroheptane sulfonate (PFHpS)

Perfluorodecanoic acid (PFDA)

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PUBLICATIONS

1. **Liew Z,** Ritz B, Rebordosa C, Lee PC, Olsen J. Acetaminophen use during pregnancy, Behavioral Problems, and Hyperkinetic Disorders. JAMA Pediatr. 2014;168(4):313-320.
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PRESENTATIONS

1. The European Congress of Epidemiology 2013
2. Society for Epidemiologic Research 2013, 2014
3. International Society of Environmental Epidemiology 2013, 2014

Chapter I. Introduction and Background

1.1 Perfluoroalkyl Substances and the potential impact on fetal brain development

Perfluoroalkyl Substances (PFASs) or Perfluorinated Chemicals (PFCs) are synthetic compounds made of carbon and fluoride atoms which have been manufactured since the 1950s. They are used extensively in industry and commercial products, such as for protective coating on food-contact packaging, and in textiles, carpets, pesticides, and personal care products because of their unique properties that make materials stain, oil, and water resistant. PFASs are extremely resistant to environmental and metabolic degradation and have been detected globally in the environment and wildlife (Lau et al. 2007) .

Perfluorooctanoic acid (PFOA) and perflourooctanesulfonate (PFOS) are the two most studied and widely used PFASs, following by perfluorohexane sulfonate (PFHxS) and perfluorononanoic acid (PFNA) (Houde et al. 2006). The different classification and origins of various PFASs have been introduced in details previously (Buck et al. 2011). Contamination of food from packaging, bioaccumulation in the food chain, and household dust were suggested as the major routes of PFAS exposure in human (D'Eon J and Mabury 2011). The biological half-lives were estimated about 4-5 years for PFOS and PFOA and 7-8 years for PFHxS in human (Olsen et al. 2007). In the U.S general population, PFOA, PFOS and PFHxS were detected in all (100%) serum samples from the 1999-2000 National Health and Nutrition examination Survey (NHANES) whereas PFNA was detected in 95% of collected samples (Calafat et al. 2007a). The trends in exposure to PFASs were recently examined using NHANES 1999-2008 serum samples (Kato et al. 2011), and the study found PFOS concentrations decreased since 2000 because of discontinuing industrial production of PFOS, but PFNA concentrations showed a significant

upward trend. PFOA concentrations during 1999-2000 were higher than during any other time period examined, but PFOA concentrations have remained essentially unchanged during 2003-2008. PFHxS concentrations showed a downward trend from 1999 to 2006, but concentrations increased during 2007-2008. Furthermore, exposure to other short-chain compounds such as perfluorobutane sulfonate (PFBS) is reported to be increased (Glynn et al. 2012) indicating industries had replaced PFOS with alternative compounds that have yet been studied and with limited data on their potential health influences.

Environmental exposures that occur during fetal development or early childhood can lead to long-term health risks that may manifest later in life. Fetuses and newborn children may be exposed to PFASs that cross the placenta barrier (Gutzkow et al. 2012) or via the mother's breast milk (Brantsater et al. 2013). Experimental studies suggested PFASs are neurotoxic, and may induce neurobehavioral effects particularly in developmentally exposed animals, via mechanism such as affect the thyroid system, influence the calcium homeostasis, protein kinase C, synaptic plasticity and cellular differentiation (Lau et al. 2007, Lau, Butenhoff and Rogers 2004, Mariussen 2012). For examples, in-utero exposure to PFOS in rat models was linked to a reduction in thyroid hormone which is known to regulate brain development (Lau et al. 2003, Luebker et al. 2005). Other animal studies found developmental neurotoxicity manifesting in motor function change and delayed learning (Fuentes et al. 2007a, Fuentes et al. 2007b, Johansson, Fredriksson and Eriksson 2008) or changes in spontaneous behavior and habituation ability (Johansson et al. 2008). Also, neonatal exposure to PFOS and PFOA has been associated with changes in proteins like tau and synaptophysin that play an important role in normal brain development (Johansson, Eriksson and Viberg 2009).

1.2 Autism Spectrum Disorders (ASD) / Infantile Autism

ASD are neurodevelopmental disorders that are characterized by impairments in communication and reciprocal social interaction, and coupled with repetitive behavior typically manifest in children 3 years of age or more (Tamiji and Crawford 2010). Autism Spectrum Disorders is a collective term for autism-related disorders. Below table 1.2.1 shows the comparison of ASD and its subtypes by classification of the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) (APA 2010) or the International Statistical Classification of Diseases, tenth revision (ICD-10) (Organization 2010).

Table 1.2.1 ASD/PDD and its subtypes by classification of DSM-IV or ICD-10

Autism Spectrum Disorders (ASD) / Pervasive Developmental Disorders (PDD)	
DSM-IV	**ICD-10**
299.00 Autistic Disorder	F84.0 Infantile Autism
299.80 Rett's Disorder	F84.2 Rett's Syndrome
299.10 Childhood Disintegrative Disorder	F84.3 Childhood Disintegrative Disorder
299.80 Asperger's Disorder	F84.5 Asperger's Syndrome
299.80 Pervasive Developmental Disorder not otherwise specified (PDD-NOS) (including Atypical Autism)	F84.1 Atypical Autism F84.8 Other pervasive developmental disorders F84.9 Pervasive developmental disorders, unspecified F84.4 Overactive disorder associated with mental retardation and stereotyped movements

There are no diagnostically informative biomarker tests for ASD, thus the current diagnostic criteria are all based on behavioral assessments including specific types and levels of impairment in several domains (Castro and Pinto 2013). The refinement of tools for diagnostic purposes in routine clinical practice and in research is ongoing (Tateno et al. 2011, Lord and Jones 2012). It has been suggested that the spectrum nature of symptomology does not imply a single underlying etiology and that the wide range of symptoms could be explained by multiple etiologies with overlapping impairment profiles (Newschaffer et al. 2007). In this study, the term

autism refers to Infantile Autism since our cases are children who received a diagnosis of Infantile Autism according to ICD-10 codes F84.0.

The incidence of autism has strongly increased over the last decades and it generally affects boys four times more often than girls (Fombonne 2003). The prevalence of childhood autism in the US recently has been reported to be as high as 40-60 in 10,000 (Duchan and Patel 2012, Hertz-Picciotto and Delwiche 2009) with marked increases over past decades (Hertz-Picciotto and Delwiche 2009, King and Bearman 2009, Hertz-Picciotto 2009). However, whether the rise in autism can be fully explained changes in diagnostic criteria and by younger age at diagnosis remain open to debate (Newschaffer et al. 2007, Hertz-Picciotto 2009).

The factors contributing to autism are not well understood. Some perinatal risk factors have been suggested such as fetal distress, birth injury, multiple birth, low birth weight, congenital malformations and pregnancy complications in general (Guinchat et al. 2012, Gardener, Spiegelman and Buka 2011). In addition, older parental age (Guinchat et al. 2012) and endocrine factors (Tareen and Kamboj 2012) have also been proposed. A strong genetic component involving multiple genes for familial autism has been reported (Geschwind 2011). A recent review suggested that as many as 2193 genes, 2806 SNPs/VNTRs, 4544 copy number variations and 158 linkage regions may be associated with autism, with 434 rated "high-confidence" genes identified (Xu et al. 2012). Despite the much larger scientific focus on genetic influences to date, it is likely that environmental factors also contribute to autism (Keller and Persico 2003, Herbert et al. 2006). A recent well-designed twin study indicated that shared environmental factors may explain 55% of model variance in prediction of autism (37% explained by genetic heritability), a much larger role than previously thought (Hallmayer et al. 2011). However, thus far very few environmental risk factors have been identified in epidemiologic studies, which have been

limited with regard to sample size and valid exposure assessment tools. An exception to this is a recent large study conducted in Los Angeles California that linked traffic-related air pollution during pregnancy to autism in ~8000 children (Becerra et al. 2012). Moreover, two much smaller case-control studies from the San Francisco Bay area and North Carolina suggested that exposures to some air toxics such as mercury, cadmium, nickel, trichloroethylene, vinyl chloride, methylene chloride, quinoline, and styrene may be associated with autism (Windham et al. 2006, Kalkbrenner et al. 2009). A recent study attempted to examine the associations between 52 different endocrine disrupting chemicals including PFASs and autistic behaviors in children but no conclusive evidence was found probably due to small sample size (175 mothers and children) and low statistical power. Further studies that examine samples to evaluate the potential link between PFASs and autism in children are needed.

1.3 Attention-Deficit/Hyperactivity Disorder (ADHD)

ADHD is one of the most common neurobehavioral disorders affecting approximately 3-5% of school-age children worldwide (Polanczyk et al. 2007). ADHD is characterized by developmentally inappropriate inattention, hyperactivity, increased impulsivity and motivational/emotional dysregulation. ADHD also disproportionately impacts boys compared with girls (Arnold 1996). Studies that compared the differences of ICD-10 and DSM-IV diagnoses have found that in Europe only children with the most severe DSM-IV diagnosed ADHD combined type (both inattention and hyperactivity/impulsivity symptoms) receive a diagnosis of Hyperkinetic Disorder (HKD) or ADHD under ICD-10 diagnostic criteria (Faraone et al. 2003, Lee et al. 2008).

Table 1.3.1 ADHD/HKD and its subtypes by classification of DSM-IV or ICD-10

Attention-Deficit/Hyperactivity Disorder (ADHD) / Hyperkinetic Disorder (HKD)	
DSM-IV	**ICD-10**
314.01 ADHD, Combined type	F90.0 Disturbance of activity and attention (ADHD, attention deficit syndrome with hyperactivity)
314.00 ADHD, predominantly Inattentive Type	F90.1 Hyperkinetic conduct disorder
314.01 ADHD, predominantly Hyperactiv Impulsive Type	F90.1 Other hyperkinetic disorders
	F90.9 Hyperkinetic disorder, unspecified

Although considerable research has been devoted to identifying factors that contribute to ADHD, its etiology is still not well understood. Emerging evidence indicates that many neurodevelopmental disorders, including ADHD, may result from complex interactions of genetic, environmental, and social factors (Pennington et al. 2009). Family studies have consistently found higher rates of ADHD in parents and siblings of affected children compared with rates among relatives of unaffected controls (Biederman 2005). Low birth weight and prematurity have repeatedly been linked to ADHD risk (Aarnoudse-Moens et al. 2009). Other maternal lifestyle factors such as prenatal exposure to nicotine, alcohol, psychosocial stress during pregnancy were also suggested to increase the risk of ADHD in children (Linnet et al. 2003). A recent review provided a synthesis of findings and listed prenatal PCB exposure as being associated with deficits on tasks used to assess neurodevelopmental functions that are deficient in ADHD children (Boucher, Muckle and Bastien 2009). Furthermore, one study described greater vulnerability for AHDH in conjunction with prenatal PCB exposure in children who were not breast fed (Jacobson and Jacobson 2003). In addition, more complex models for the etiology of ADHD that incorporate gene-environment interplay have also been proposed. A study focused on the joint effects of dopamine pathway-related genes and prenatal substance exposures and found that smoking during pregnancy is associated with ADHD in genetically

susceptible children (Neuman et al. 2007) and another study reported organophosphate exposure to be related to ADHD prevalence (Kuehn 2010).

In early 2010, two large cross-sectional studies reported a positive correlation between PFASs exposure and diagnoses of ADHD in children. Utilizing data from NHANES, the odds of ADHD among US children 12-15 years of age was found to be increased in children with higher serum PFASs levels including PFOS, PFOA, PFHxS, and PFNA (Hoffman et al. 2010). Another report using cross sectional data from the C8 Health project, a 2005-2006 survey conducted in a Mid-Ohio valley community that was highly exposed to PFASs through contaminated drinking water, also reported that children with higher serum level of PFOS and PFHxS had a higher odds in having an ADHD diagnosis (Stein and Savitz 2011). However, these studies suffer from several limitations including the possibility of reverse causality due to the cross-sectional survey design, as well as potential outcome misclassification since they relied solely on parent reported ADHD diagnoses, in addition to potential selection bias due to non-participation. Two prospective studies later reported prenatal exposures to PFOS and PFOA were not associated with behavioral problems in 7-year-old children in Denmark (Fei and Olsen 2011b), and PFOA decreased ADHD characteristics among children aged 6-12 years in the US (Stein, Savitz and Bellinger 2013); the outcome of these studies relied on behavioral screening instead of clinical diagnoses. The questions of whether exposures to PFASs are associated with ADHD remain unclear and further prospective studies are urgently needed.

1.4 Cerebral Palsy (CP)

CP is a group of disorders affecting the development of body movement, muscle coordination, and posture; specifically resulting from non-progressive lesions in the fetal or neonatal brain, or of the immature central nervous system (Bax et al. 2005). CP is the most

common neurological disease that will lead to severe physical disability and it affects about 2-3 of 1000 births (Koman, Smith and Shilt 2004, Kuban and Leviton 1994). The majority of people with cerebral palsy are born with it and early signs usually appear before the age of 3 years (Pakula, Braun and Yeargin-Allsopp 2009). More than 50% of children with CP cannot walk independently and use assistive devices such as walkers or wheelchairs, and 70% have another major developmental disability, primarily mental retardation or vision impairment (SCPE 2000).

Due to the complexity of the disorder and the diversity of the symptomatology, CP is not defined as a case (a reliable diagnosis) until a child's motor symptoms typically stabilize by 2-3 years of age. For CP diagnosis, doctors use motor skills test to evaluate a child's muscle development and tone, and unusual posture, and computed tomography (CT) scans and magnetic resonance imaging (MRI) to image the brain and determine underdeveloped or damaged areas of brain tissue (Tomasovic and Predojevic 2011). By evaluating a medical history, doctors can rule out other conditions, by checking if the child's symptoms are non-progressive, and characteristic of CP. Manual ability are often discrepant in children with CP and patterns vary across subgroups; therefore, the case definition of CP is defined both in terms of distribution of the motor disorder (limbs affected), and the site of the brain lesion (Uldall et al. 2001).

CP has been found to be much higher in preterm births, up to 100 cases per 1000 births in extreme pre-term cases (<28 weeks) (O'Shea 2008). Improvements in perinatal care and neonatal survival in recent decades have increased the survival of children born preterm, and therefore the number of CP cases born preterm also increased (Paneth, Hong and Korzeniewski 2006, O'Shea 2008). Postnatal factors such as head trauma or birth asphyxia have traditionally been assumed to be the cause of CP, but these factors only appear in less than 10% of CP cases (Clark, Ghulmiyyah and Hankins 2008). Among the prenatal risk factors, maternal intrauterine infection

(defined as chorioamnionitis which is an inflammation of the fetal membranes a bacterial infection) is the best known (Bashiri, Burstein and Mazor 2006, Shatrov et al. 2010). Several other studies have suggested a role for disorders (possibly genetic) in inflammatory response to the infection and disorders in coagulation (including inherited thrombophilias) in CP development, but whether these associations are causal is not known and the pathway is still poorly understood (Nelson 2008, Nelson et al. 1998).

Several prenatal and perinatal factors such as multiple birth, preterm birth, low birth weight, and fetal infection have been associated with CP (McIntyre et al. 2012). Moreover, environmental factors that may cause brain lesions in early life was suggested may also play a role in the etiology of CP (Green and Hurvitz 2007, Koman et al. 2004, Kuban and Leviton 1994). However, to date still very little is known about possible environmental causes of CP. Severe stress in prenatal life was found to increase the susceptibility for CP (Li et al. 2009). Prenatal exposure to methylmercury (Gilbert and Grantwebster 1995, Gilbertson 2004), lead and polychlorinated biphenyls (Winneke 2011), carbon monoxide (Alehan, Erol and Onay 2007) were suggested may be associated with CP, but no evidence is conclusive. To our knowledge, associations between prenatal PFAS exposure and CP have not been investigated previously.

Chapter II. Databases

2.1 The Danish National Birth Cohort

The Danish National Birth Cohort (DNBC) (Olsen et al. 2001) is a nation-wide follow-up study of pregnant women and their offspring in Denmark with the purpose to investigate the effects of early life exposures, from conception to early childhood, on the health of the offspring. Mothers and live-born children are considered two cohorts to be followed for decades to examine the occurrence of diseases and well-being in a life-course approach. Recruitment for the DNBC took place from 1996-2002 and a total of 101,042 pregnancies were initially enrolled. General practitioners in Denmark invited women at their first pregnancy visit, usually during weeks 6-12 of pregnancy, to participate in the study. More than 95% of women in the study were recruited based on the invitation received at their first general practitioner visit. The remaining subjects were referred by midwives as a secondary recruitment procedure. The only exclusion criteria were not having access to a telephone, not speaking Danish well enough to complete the interview, and not intending to carry the pregnancy to term. About 60% of all women invited accepted and signed the informed consent form.

Women were contacted by trained female interviewers who conducted computer-assisted telephone interviews based on highly structured questionnaires. The first set of interviews was conducted four times at gestational weeks 12 and 30, and when the child was 6 and 18 months old. Interviews were considered missing if women were not reached at the scheduled time or after three additional attempts to make contact. Interviews were cancelled if the woman was no longer pregnant when contacted prior to giving birth. Two maternal blood samples taken during pregnancy, and one umbilical cord blood sample obtained at birth were obtained and stored in a

biobank. All Regional Science Ethics Committees in Denmark have approved the DNBC. English version of all the questionnaires can be found online at http://www.bsmb.dk.

Data from the DNBC can be linked to several Danish national registries via a unique personal identification number in order to obtain follow-up health and covariate information not provided in the DNBC interviews (Pedersen et al. 2006).

2.2 The Medical Birth Registry

The Medical Birth Registry (Knudsen and Olsen 1998) was established in 1968 and has been computerized since 1973. It contains data on all live births and stillbirths in Denmark, including characteristics of mother and child and data on pregnancy and delivery. Its initial purpose was to monitor the health of newborns and quality of antenatal and delivery care services. It contains data on such factors as gestational age, birth weight, and Apgar score at 5 minutes after delivery.

2.3 The Danish National Hospital Register

The Danish National Hospital Register (Andersen et al. 1999)contains information on all discharges from Danish hospitals since 1977. Outpatient data has been included in the register since 1995. Diagnostic information is based on the Danish version of the International Classification of Diseases, eighth revision (ICD-8), and tenth revision (ICD-10) from 1994 onwards. All treatments in Danish hospitals are free of charge for all residents.

2.4 Danish Psychiatric Central Registry

The Danish Psychiatric Central Register (Munk-jorgensen, Kastrup and Mortensen 1993) contains information dating from the 19th century; data were collected systematically from 1938. As of 1969 data on psychiatric admissions have been computerized and include all admissions to psychiatric hospitals and psychiatric wards in Denmark. Since January 1, 1995 information about

all psychiatric outpatient contacts have also been included. Diagnostic information is based on the Danish version of the International Classification of Diseases, eighth revision (ICD-8), and tenth revision (ICD-10) from 1994 onwards.

2.5 Danish National Cerebral Palsy Register

The Danish National Cerebral Palsy Registry (DNCPR) (Topp, Langhoff-Roos and Uldall 1997, Uldall et al. 2001) in Denmark was started in 1967 and includes cases born after 1950. The register is population-based and originally covered only Eastern Denmark or about 50% of the Danish population, in 1992 the register became a public national registry and was expanded to cover the entire Danish population. The DNCPR is based on voluntary reports from all 14 pediatric departments and three special institutions for disabled children in Denmark. The voluntary reports are combined with information from The Danish National Hospital Register in order to ensure completeness of the registry. Lists from the National Hospital Register are produced every half year and sent to the departments and institutions for follow-up on additional possible CP cases. Neuropediatricians at each reporting institution submit a copy of the medical record for each identified potential case to the DNCPR. Children are included in the register if they have CP, defined as "a disorder of movement and posture due to a defect or lesion of the immature brain, excluding disorders which are of short duration, due to progressive disease or due solely to mental deficiency". Inclusion in the DNCPR is based on information about the children at the age of 4-6 years. Children who died between the age of 1 and 5-6 years are included if the CP-diagnosis is unquestionable and if their diagnosis is expected to have a pre- or peri-natal etiology (event occurring before 28 days of age). Before inclusion in the register, the diagnosis is confirmed by at least two neuropediatricians.

Chapter III: Study Aims, Design, and PFAS measurement

The main study hypothesis is that prenatal exposure to perfluorinated chemicals affects fetal neurodevelopment and increases the risk of Childhood Autism, Attention-Deficit/Hyperactivity Disorder or Cerebral Palsy in children.

Specific aims:

a) Report descriptive statistic of PFASs concentration in maternal plasma with demographic characteristic of mothers and children, and correlations among various PFASs.
b) Investigate the associations between prenatal PFASs exposures and the risks of autism, ADHD, and CP.
c) Under the framework of this study, conduct a bias analysis to examine whether conditioning on live-born status may induce bias in birth cohort studies.

3.2 Study design and subject selection

We employed a case-cohort design, a type of case-control study, where a common group of controls is randomly selected from a defined source population as a comparison group for multiple outcomes (Rothmand KL 2008). 101,042 pregnancies were originally enrolled in DNBC from 1996 to 2002. The source population for this study is defined as all singletons in DNBC born alive and who are at risk of receiving one or more of the diagnoses (autism, ADHD, CP) under study. Furthermore maternal blood samples collected during first or second pregnancy trimesters needed to be available and women had to be taking part in the first telephone interview. We excluded women with unsuccessful pregnancies (n=6,207), multiple births other than singleton (n=2,080), unknown birth outcomes (n=80), missing dates of birth (n=99), and those who missed either the first telephone interview or did not have a maternal blood sample available

(n=9187); thus, a total of 83,389 mother-children (42,737 boys and 40,652 girls) comprise our source population.

From this source population, we randomly selected 220 among 890 children who have been diagnosed with Attention-Deficit/Hyperactivity Disorder (ICD10 F90.0), and 220 among 301 children that have been diagnosed with Infantile Autism (ICD10 F84.0) before the end of follow up of August 2011. Diagnoses of ADHD and Autism were identified from the Danish National Hospital Registry (NPR) (Andersen et al. 1999) and the Danish Psychiatric Central Registry (PCR) (Munk-jorgensen et al. 1993) relying on Danish residents' unique civil registration number for linkage. ADHD and Autism diagnostic information is based on the International Classification of Diseases, 10^{th} version as primary or secondary diagnoses (97.5% of identified cases received a primary diagnosis). We further selected all 156 children in the DNBC who received a diagnosis of cerebral palsy according to the Danish National Cerebral Palsy Register (DNCPR).

As controls, we selected at random 550 children (110 females and 440 males) from the entire source population after frequency matching on sex. In addition, we over-sampled 50 controls at random from 3,866 children who were born preterm (before 37 weeks of gestation).

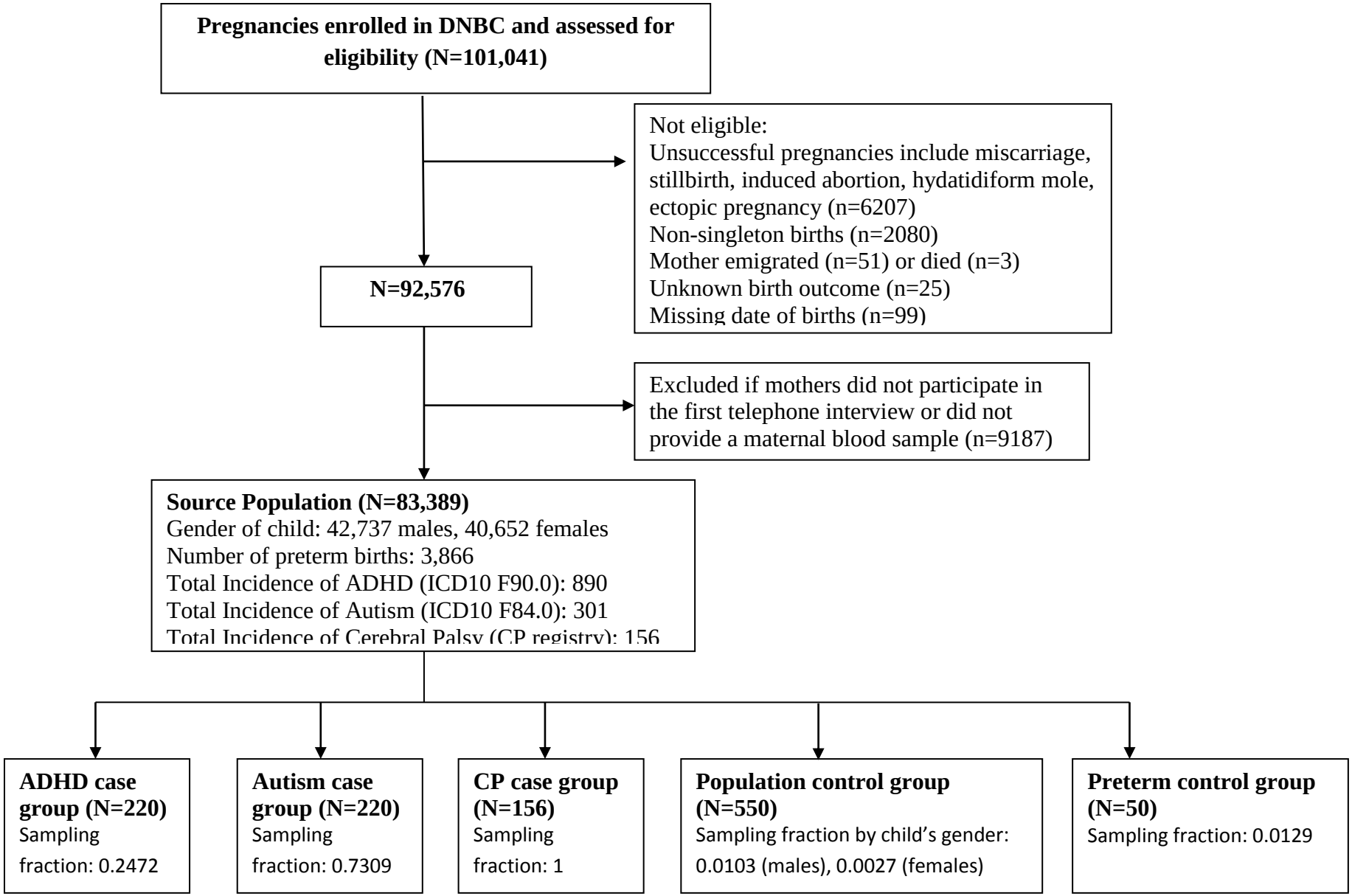
Table 3.2.1 Cases sampling fractions from the source population

Outcome	NPR (n)	PCR (n)	male (n)	females (n)	Total diagnoses (n)	Selected Sample (n)	Sampling fraction
CP (DNCPR)	n/a	n/a	88	68	156	**156**	1.0
Autism (ICD10 F84.0)	15	286	253	48	301	**220**	0.7309
ADHD (ICD10 DF90.0)	99	791	726	164	890	**220**	0.2472

Table 3.2.2 Control sampling fractions from the source population

Control type	Source population (N)	Selected controls	Sampling fraction
Population controls	42737 males , 40652 females	400 males, 110 females	0.0103 males, 0.0027 females
Pre-term controls	3866	50	0.0129

Figure 3.1 Flow chart of study population selection in the FETOTOX sub-study4.



3.3 PFAS measurement

PFAS concentrations in maternal plasma were analyzed at Aarhus University, Department of Environmental Science, under supervision of senior scientist Rossana Bossi. Solid Phase Extraction technique was used for PFASs extraction and purification from plasma samples, and PFASs concentrations were measured by liquid chromatography–mass spectrometry (LC-MS/MS).

Solid-phase extraction is a separation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties. Solid phase extraction can be used to isolate analytes of interest from a wide variety of matrices, including urine, blood, water, beverages, soil, and animal tissue (Supelco 1998).

Liquid chromatography–mass spectrometry (LC-MS/MS) is a chemistry technique that combines the physical separation capabilities of liquid chromatography with the analysis capabilities of mass spectrometry (Arpino 1992). LC-MS/MS is a commonly used technique for detection and identification of chemicals in a complex mixture in which various chemicals are present. Mass spectrometry is an analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structures of molecules, such as peptides and other chemical compounds. MS works by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios.

We used 0.1 ml from each stored maternal plasma sample for PFASs analyses. When samples were identified and taken out of the freezers, they are placed in a random order and by

blocks that match the lab procedures (24 samples per batch). Each block contains a proportion of cases and controls matching the proportion in the overall sample. All samples were given a random number from a list of 4 digit random numbers before being sent to the lab.

A total of 1,400 randomly selected maternal plasma samples in DNBC had PFASs levels analyzed in previous studies (Fei et al. 2007b, Fei 2009). For quality control, we additionally sampled 6 blood samples (2 with low PFAS values, 2 with average values and 2 with high values) that were not part of the study, but had previously been analyzed at the 3M Toxicology Laboratory (St. Paul, MN) with similar analytical techniques. The overlapping samples measurement from previous and current study would allow us to check for correlations between PFAS values analyzed from two different laboratories.

Chapter IV: Prenatal Exposure to Perfluoroalkyl Substances and Risk of Congenital Cerebral Palsy in Children

4.1 Abstract

Perfluoroalkyl substances (PFASs) are persistent pollutants and endocrine disruptors that may affect fetal brain development. We investigated whether prenatal exposure to PFASs increases the risk of congenital cerebral palsy (CP). The source population of this study includes 83,389 liveborn singleton children and mothers enrolled in the Danish National Birth Cohort (DNBC) during 1996–2002. We identified 156 CP cases by linking the cohort to the Danish National Cerebral Palsy Register and we randomly selected 550 controls using a case-cohort design. Sixteen PFASs were measured in maternal plasma samples collected in early or mid-pregnancy, and Six PFASs were quantifiable in more than 90% of the samples. We found higher risks of CP in boys with increasing maternal PFAS levels (RR=1.7 (95%CI 1.1-2.9) per one unit (natural-log ng/mL) increase in perfluorooctane sulfonate (PFOS) and RR=2.0 (95%CI 1.2-3.4) per unit increase in perfluorooctanoic acid (PFOA)). We also observed a dose-response pattern of CP risks in boys per PFOS and PFOA quartile (p-trend<0.01). PFAS was associated with both spastic unilateral or bilateral CP sub-phenotypes. No association between PFASs and CP was found in girls. Prenatal exposures to common PFASs may increase the risk for CP in boys, but the finding is novel and replication is needed.

4.2 Introduction

Congenital cerebral palsy (CP) are permanent and non-progressive movement and posture disorders attributed to early brain lesions (Rosenbaum et al. 2007). CP affects about 2-3 per 1000 births (Ravn, Flachs and Uldall 2010, Clark et al. 2008), with an incidence as high as 100 cases per 1000 births among extremely preterm births (Vincer et al. 2006). More than half of the

children affected by CP are unable to walk without assistive devices, or have a co-morbidity such as mental retardation or vision impairment (SCPE 2000). Birth asphyxia or trauma are present in less than 10% of cases (Clark et al. 2008), and the etiology of the majority of CP cases remains unexplained (Marret, Vanhulle and Laquerriere 2013) .

Perfluorinated chemicals (PFASs) are a group of synthetic chemicals extensively used in food packaging, non-stick pan coatings, fire-fighting foams, paper and textile coatings, and personal care products. PFASs have surfactant properties and are extremely persistent in the environment (Houde et al. 2006). Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) were in the study period the two most frequently used PFASs in Denmark with biological half-lives of 4 to 5 years (Olsen et al. 2007). After a drop in manufacturing emission in 2002, PFOS levels in humans were reported to decrease in some countries, however exposure to substitute PFASs such as perfluorobutane sulfonate (PFBS) and perfluorononanoic acid (PFNA) have subsequently increased (Glynn et al. 2012).

While the low level of PFAS found in adult population may cause no harm, concerns about potential health consequences of PFAS exposure in fetal life have been raised. PFAS can cross the placental barrier (Fei et al. 2007a), affect neuronal cell development (Johansson et al. 2009) , change motor function and lead to delayed learning in animals (Johansson et al. 2008, Onishchenko et al. 2011) . In addition, PFASs have endocrine disruptive properties (Kjeldsen and Bonefeld-Jorgensen 2013) and can interfere with thyroid hormone function (Wen et al. 2013, Lin et al. 2013, Lau et al. 2003, Long, Ghisari and Bonefeld-Jorgensen 2013) which during fetal development may cause mental retardation and neurological deficits (Porterfield 2000).

Thus we hypothesized that prenatal PFAS exposure affect fetal brain development and increase the risks for CP. Within the framework of the Danish National Birth Cohort (DNBC), we measured PFAS concentrations in maternal plasma collected prenatally to examine children's risk of developing CP.

4.3 Methods

We performed a case-cohort study utilizing data from the DNBC described in detail previously (Olsen et al. 2001). Briefly, from 1996 to 2002 pregnant women were recruited through their general practitioners during gestational weeks 6 to 12. About 50% of all general practitioners in Denmark participated in the study, and 60% of invited women agreed to participate. Women were ineligible if they did not speak any Danish or intended not to carry their pregnancy to term. Information was collected in four computer-assisted telephone interviews (twice during pregnancy and twice postpartum), and two maternal blood samples were taken, one each in the first and second trimester (English versions of questionnaires are available online at http://www.bsmb.dk.)

The study is part of the FETOTOX program (http://www.fetotox.au.dk) that examines neurodevelopmental influences of prenatal exposures to PFASs, and we present our findings for CP in this report.

Source population

The DNBC source population for this study was 1) live born singletons at risk of CP; 2) infants born to women who participated in the first telephone interview conducted approximately during the 12^{th} gestational week; 3) infants of mothers from whom we collected blood during the first or second pregnancy trimesters. From 101,041 pregnancies originally enrolled in the DNBC,

we excluded unsuccessful pregnancies (n=6,207), non-singleton births (n=2,080), births with unknown birth outcomes (n=25) or missing birth dates (n=99), mothers who emigrated (n=51) or died (n=3), and women who missed the first telephone interview (n=4578) or did not provide a pregnancy blood sample (n=4609). This left us with 83,389 mother-child pairs (42,737 boys and 40,652 girls) as the source population.

Congenital Cerebral Palsy

We identified 156 children in the DNBC source population who were diagnosed with CP according to the Danish National Cerebral Palsy Register (DNCPR). The linkage of DNBC and DNCPR was updated up to September 23, 2010. The DNCPR is a population-based registry that contains records of individuals with validated CP diagnosis since 1925 (Uldall et al. 2001, Ravn et al. 2010). The register has followed the definition from the Surveillance of Cerebral Palsy in Europe (SCPE 2000) defining CP as a group of permanent movement and/or posture disorders due to non-progressive interference/lesion/abnormality in the developing/immature brain since 1978. The motor disorders described as unilateral or bilateral manifestations, and the main affected brain areas are in the white matter (spasticity), basal ganglia (dystonic/dyskinetic), or cerebellum (ataxia). The distributions of clinical subgroups of the 156 CP cases in this study are: 137 spastic, 13 dystonic, 2 ataxic, 2 choreatic and 2 unclassified. Cases of CP are validated by a child neurologist and an obstetrician based on reviews of the child's medical records and information collected from all hospitals and pediatric departments in Denmark.

Controls selection

We used a case-cohort design and randomly selected 550 children (110 females and 440 males) by sex from the source population in the population control group. More male than

female controls were sampled because the population control group was also designed as a comparison group for other outcomes we targeted including attention-deficit/hyperactivity disorder and autism both diseases 4 times more prevalent in boys. In addition, we over-sampled an additional 50 controls at random from 3,866 children who were born preterm (before 37 weeks of gestation) but we only used the preterm controls for secondary analyses. The flowchart of subjects' selection and sampling fractions of cases and controls are shown in Figure 3.1 in Chapter III.

PFAS exposure

Plasma concentrations of perfluorinated chemicals were analyzed at the Department of Environmental Science at Aarhus University. In the DNBC, two maternal blood samples were collected and sent by mail to Statens Serum Institut in Copenhagen, and then separated and stored in freezers at -80°C or in liquid nitrogen. Blood samples were transported and subjected to outdoor temperatures for 4-48 hours, but most samples arrived within 28 hours. We used 0.1 ml stored maternal plasma sample for PFAS analyses; 86% of samples for both cases and controls were collected at the first antenatal visit during the first trimester, and 14% at the second antenatal visit in the second trimester.

Solid Phase Extraction (SPE) technique was used to extract and purify PFAS from plasma samples, and PFAS concentrations were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS). All samples were measured in a random sequence of case or control status by laboratory personnel blinded to participant information. Nine maternal plasma samples (4 CP, 5 population controls) were either not available from the biobank or failed the PFAS extraction and purification process thus were excluded. For quality control, we analyzed 6 blood

samples (two each with low, average and high concentrations of PFOS/PFOA) that were not part of this study but had previously been analyzed at the 3M Toxicology Laboratory with similar analytical techniques (Fei et al. 2007a, Ehresman et al. 2007). In addition, 15 samples that included in this study had also previously been analyzed in the 3M laboratory (Fei et al. 2008, Fei et al. 2007a). Correlations between PFOA and PFOS values measured at the two laboratories were compared.

Statistical analysis

PFAS concentrations were analyzed as continuous variables (with or without natural-log transformation) as well as categorized into quartiles according to the distribution among population controls; the lowest quartile was used as reference group. We used generalized linear models with a log link function based on a Poisson distribution to estimate risk ratios (RR) and 95% confidence intervals (CI) for PFAS exposures and CP, taking into account inverse probability weights (IPW) derived from the sampling fractions of cases and controls. Trend tests were performed using median values of PFAS concentrations in each quartile as a continuous variable.

In addition, we fit generalized additive models to examine potential non-linear relation between maternal plasma PFAS and CP with a smoothing function of PFAS concentrations without imposing a given parametric form. Five knots was set as the upper limit of the number of degrees of freedom, and we compared model fit and visually inspected plots of the smoothed data. We did not find evidence for non-linearity between PFAS values and CP.

Since PFASs are hormonally active, we conducted separate analyses for boys and girls. We also performed stratified analysis by term and preterm birth status; in this analysis we added the

preterm controls additionally selected from the DNBC. We also evaluated the relation of PFAS by subtype of spastic CP (unilateral or bilateral manifestations).

Final models were adjusted for potential confounders that were previously described as risk factors of CP or factors associated with PFAS exposures including maternal age at child's birth, parity, socio-economic status (SES; derived from the mother's and father's education level and occupation.), smoking and alcohol drinking during pregnancy, and mother's self-reported psychiatric illnesses, in addition to gestational week of blood drawn and child's sex. To determine maternal psychiatric illnesses, women were asked if they had seen a doctor or psychologist because of depression, anxiety, childhood psychiatric disorder, family problems/life crisis, or other mental health problems Additionally, other potential confounders such as child's birth year, father's age at child's birth, mother's pre-pregnancy body mass index, season of conception, maternal fever or infections during pregnancy were evaluated but not included in final models as they changed effect estimates of interest by less than 1%. Using propensity scores, we further adjusted for some dietary factors (e.g. fish and organic food consumption) and household attributes (e.g. ownership, size) that may be common sources of exposure to PFASs and other endocrine disrupting compounds such as bisphenol A and phthalates (Ye et al. 2009, Casas et al. 2013).

We focus on six out of sixteen different PFASs with the following proportions of samples above the lower limit of quantitation (LLOQ): PFOS 100% , PFOA 100%, PFHxS (perfluorohexane sulfonate) 98%, PFHpS (perfluoroheptane sulfonate) 96%, PFNA 92%, PFDA (perfluorodecanoate) 90% (see full PFAS panel with LLOQ in Supplementary Table S4.1). To account for missing PFAS values <LLOQ when PFASs were analyzed as continuous variables, we used multiple imputations (Lubin et al. 2004) with the procedures "PROC MI" in SAS

including six PFASs and all covariates in the model. Five simulated complete datasets were generated after imputation, and we employed standard analytical procedures to combine the results.

Correlations between PFAS concentrations were assessed based on a Pearson correlation matrix (Supplementary Table S4.2). To examine whether any single PFAS may be of particular importance, we simultaneously included all PFASs in one model. To ensure that individuals with extreme exposure values did not disproportionately influence our results, we conducted sensitivity analyses excluding observations greater than three times the 75th percentile for each PFAS. Analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

4.5 Results

Table4.1 presents the demographic characteristics of the participants. Table 4.2 shows the median and inter-quartile range of maternal plasma PFASs concentrations in cases and controls separately for boys and girls. For quality control, we found a high correlation between the PFOS/PFOA levels measured in current study and previous measurements performed at the 3M laboratory (Pearson correlation r=0.94 for PFOS and r=0.95 for PFOA).

We estimated increased risk for CP per natural-log increase in maternal PFOS, PFOA, and PFHpA in boys, and in term born boys (Table 4.3). Positive associations between PFAS concentrations and CP in boys were found for both spastic unilateral and bilateral sub-phenotypes of CP (Supplementary Table S4.3). We found no associations between PFASs and CP in girls or in term born girls (Table 4.3). Results are very imprecise for boys born preterm (ie. per natural-log unit increase in PFOS RR=1.1 95%CI 0.3-5.3, or PFOA RR=1.7 95%CI 0.3-10.0), and numbers are too sparse to perform estimations in girls born preterm.

Higher risks were observed among boys in higher quartiles of PFOS, PFOA, and PFHpS compared to the lowest (Figure 4.1). Simultaneous adjustment for all PFASs in one model weakened most PFAS and CP associations, but high PFOS concentrations remained positively associated with CP risk (Supplementary Table S4.4). Using propensity scores to additionally adjust for common sources of exposures to PFASs and other endocrine-disrupting chemicals did not remove the observed associations ie. elevated CP risks in boys among the highest quartile of PFOS (RR=2.8; 95%CI 1.4-5.4) or PFOA (RR=2.2; 95%CI 1.1-4.1) compared to the lowest. Additional sensitivity analyses comparing with or without natural-log transformation of PFAS values, and excluding extreme PFAS values from the analyses did not change our findings (data not shown).

4.6 Discussion

We found a dose-response like association between prenatal exposure to PFAS and the risks for CP in boys, and similar associations were seen for spastic unilateral or bilateral CP sub-phenotypes. No associations between PFASs and CP were found in girls, but the statistical power is low due to small numbers.

Recent evidence in animal and human studies suggest that PFASs interfere with maternal hormone function during pregnancy (Wen et al. 2013, Lin et al. 2013, Lau et al. 2003, Long et al. 2013, Kjeldsen and Bonefeld-Jorgensen 2013), and deficiency of thyroid hormone during critical periods of brain development can damage the nervous system and cause neurodevelopment disorders such as CP (Hong and Paneth 2008, Nelson and Ellenberg 1985).

Two previous studies used subsets of the DNBC samples and found no associations for prenatal exposures to PFOS/PFOA and scales of motor function and coordination of children in

age18 months (Fei et al. 2008) and 7 years (Fei and Olsen 2011a). However, the study endpoints were self-reported by mothers thus prone to measurement errors. More recently, a birth cohort from Taiwan asked trained physical therapists to evaluate children's neurodevelopment and reported higher prenatal PFOS levels associated with deceased gross-motor function in children at 2 years of age (Chen et al. 2013), but the predictive value of the used scores may be low at this young age.

Our study shows PFAS exposures correlated with CP risk only in boys thus a possible sex-specific mechanism should be further investigated. There is some evidence suggesting that PFASs are sex-specific endocrine disruptors in vitro (White, Fenton and Hines 2011) and in adults (Wen et al. 2013), and motor functions in PFAS-exposed mice were impaired in a sex-related manner (Onishchenko et al. 2011) . The vulnerability of different brain areas during different developmental windows may also differ by sex (Reiss et al. 2004) . Moreover, the male brain is suggested to be more vulnerable to white matter injuries and intraventricular haemorrhage (Johnston and Hagberg 2007) , and males are in general at higher risk of CP (Nordmark, Hagglund and Lagergren 2001) .

CP risks were elevated among boys born at term to mother with higher PFAS levels, but results are imprecise for boys born preterm. It should, however, be taken into consideration that gestational age may be a mediator in the causal pathway of PFAS and CP (Chen et al. 2012), and stratification on preterm birth status could potentially introduce collider bias that lead to either overestimate or underestimate risks (Wilcox, Weinberg and Basso 2011, VanderWeele and Hernandez-Diaz 2011).

The maternal PFAS values measured in our study are comparable to those previously reported during a similar time period in the US general population (Calafat et al. 2007b). The PFAS values <LLOQ in our studies were estimated from multiple imputations, but the influence of estimation errors would be small for PFAS quartiles. Since concentrations for several PFASs were moderately correlated, it is difficult to disentangle whether specific compounds or the combination of substances are driving the associations. PFOS remained positively associated with CP when adjusting for the other types of PFAS in the same model. A recent in-vitro assay found mixture specific effects of five PFASs tested simultaneously for their ability to interfere with androgen receptors (Kjeldsen and Bonefeld-Jorgensen 2013). Further experimental studies are needed to examine mechanisms of how different PFASs act on biologic targets.

This study has several strengths. First, PFAS values were measured in prospectively collected maternal plasma samples. PFASs have long biological half-life in human and it has previously been shown that PFAS measurement in serum or plasma samples are very comparable (Ehresman et al. 2007). High correlations between maternal and cord blood PFAS concentrations have been reported and suggest that PFAS in maternal plasma is also a valid marker of fetal exposure (Fei et al. 2007a). Moreover, participants were selected from a well-defined nationwide cohort with a sufficient duration of follow-up (~10 years on average in the DNBC) to catch all congenital CP cases, and CP cases were ascertained from records of the Danish National Cerebral Palsy Registry with unique civil registration numbers used for linkage. Since follow-up does not require subject's active participation, selection bias due to differential response is not an issue. CP diagnoses were validated by experts' review of the child's medical records which reduces disease misclassification. However, children have to survive to at least one year of age to be diagnosed in the DNCPR, therefore we likely have under-ascertained severe CP cases who

died in pregnancy, at birth, or during early infancy. If PFAS exposure reduces fetal/neonatal survival [17, 39], survival bias might occur and we would expect an attenuation of the observed results.

We have no data for other endocrine disrupting compounds and therefore we could not evaluate possible confounding by organophosphates, bisphenol A and phthalates. However, additional adjustment for common sources of exposure to endocrine disrupting chemicals such as dietary factors and household characteristics (Ye et al. 2009, Casas et al. 2013) did not change our results and conclusions. Non-participation in the DNBC cohort has been shown to have small if any effects on internal validity, but it may limit the generalizability of our results (Nohr et al. 2006).

In summary, we found that prenatal exposures to some PFASs may increase the risk for CP in children. This finding raises concerns, since PFASs are ubiquitous and persistent in the environment, and CP has long-lasting patient, care-giver, and societal impacts. This finding is novel and may lead to identification of a preventable risk factor for CP, but further studies are needed.

Table 4.1 Characteristic of study participants

Characteristic [a]	CP (N=156)		Controls (N=550)	
	n	%	n	%
Child's sex				
Male	88	56.4	440	80.0
Female	68	43.6	110	20.0
Mother age at delivery (years)				
≤ 24	18	11.5	42	7.6
25-29	58	37.2	235	42.7
30-34	51	32.7	201	36.5
≥ 35	29	18.6	72	13.1
Socio-economic status				
Low / Medium	45	28.8	209	38.0
High	111	71.2	339	61.6
Gestational age (days)				
<259	43	27.6	17	3.1
259-293	102	65.4	485	88.2
≥ 294	10	6.4	48	8.7
Parity				
1	78	50.0	247	44.9
>1	74	47.4	288	52.4
Maternal drinking during pregnancy				
Never	50	32.1	161	29.3
0-1 glass per week	37	23.7	139	25.3
> 1 glass per week	69	44.2	250	45.5
Maternal smoking during pregnancy				
Never	107	68.6	409	74.4
≤ 9 cigarettes/day	17	10.9	64	11.6
> 9 cigarettes/day	32	20.5	77	14.0
Mother's self-reported psychiatric illnesses				
Yes	39	25.0	81	14.7
No	117	75.0	469	85.3

[a] The percentages do not sum up to 100% because of missing values.

Table 4.2 Median and interquartile range of maternal plasma PFAS concentrations (ng/ml) in cases and controls, by child's sex

Perfluoroalkyl substances	Abbreviation	Carbon chain length[a]	Percentage quantifiable in all samples	Boys		Girls	
				CP (N=86)	Controls (N=435)	CP (N=66)	Controls (N=110)
Perfluorooctane sulfonate	PFOS	8	100%	28.90 (22.40-40.00)	27.60 (20.00-35.60)	27.50 (19.38-37.20)	26.20 (20.60-35.60)
Perfluorooctanoic acid	PFOA	8	100%	4.56 (3.32-6.04)	4.00 (2.98-5.34)	3.90 (3.30-5.58)	4.04 (3.14-5.50)
Perfluorohexan sulfonate	PFHxS	6	98%	0.96 (0.74-1.30)	0.93 (0.70-1.24)	0.90 (0.67-1.26)	0.92 (0.68-1.26)
Perfluoroheptane sulfonate	PFHpS	9	96%	0.49 (0.39-0.62)	0.46 (0.37-0.56)	0.42 (0.34-0.56)	0.43 (0.34-0.60)
Perfluorononanoic acid	PFNA	8	92%	0.34 (0.24-0.46)	0.31 (0.22-0.42)	0.31 (0.21-0.43)	0.31 (0.22-0.42)
Perfluorodecanoic acid	PFDA	10	90%	0.19 (0.14-0.25)	0.17 (0.14-0.24)	0.18 (0.13-0.21)	0.18 (0.13-0.23)

[a] Number of carbons in the fully-fluorinated alkyl chain.
[b] Concentrations for 9 samples (4 CP and 5 controls) were missing because samples were not available from the biobank or failed the extraction process.

Table 4.3 Risk Ratios for CP in children according to maternal PFAS concentrations during pregnancy, by child's sex and among term birth

Prenatal exposure	Boys only [a]	Girls only [a]	Term boys [b]	Term girls [b]
	Adjusted RR[d] (95% CI)	Adjusted RR[d] (95% CI)	Adjusted RR[d] (95% CI)	Adjusted RR[d] (95% CI)
Per 1 natural-log unit (ng/ml) increase				
PFOS	1.7 (1.1, 2.9)	0.7 (0.4, 1.4)	2.1 (1.2 ,3.8)	0.6 (0.3, 1.4)
PFOA	2.0 (1.2, 3.4)	0.7 (0.4, 1.4)	2.5 (1.3, 4.6)	0.6 (0.2, 1.4)
PFHxS	1.2 (0.8, 1.7)	1.1 (0.6, 2.0)	1.2 (0.8, 1.7)	0.6 (0.3, 1.2)
PFHpS	1.5 (1.0, 2.3)	0.9 (0.5, 1.5)	1.6 (0.9, 2.8)	0.8 (0.4, 1.7)
PFNA	1.3 (0.6, 2.9)	0.6 (0.3, 1.1)	1.6 (0.8, 3.1)	0.7 (0.4, 1.4)
PFDA	1.1 (0.8, 1.7)	0.7 (0.4, 1.1)	1.3 (0.8, 2.1)	0.8 (0.5, 1.3)

[a] 152 CP (86 boys, 66 girls) and 545 controls (435 boys, 110 girls) were used in analyses.
[b] 110 term CP (65 boys, 45 girls) and 530 term controls (422 boys, 108 girls) were used in analyses.
[c] Adjusted for child's sex, maternal age at delivery, SES, parity, alcohol consumption during pregnancy, smoking during pregnancy, mother's psychiatric illnesses, and gestational week of blood drawn.
[d] Adjusted for maternal age at delivery, SES, parity, alcohol consumption during pregnancy, smoking during pregnancy, mother's psychiatric illnesses, and gestational week of blood drawn.

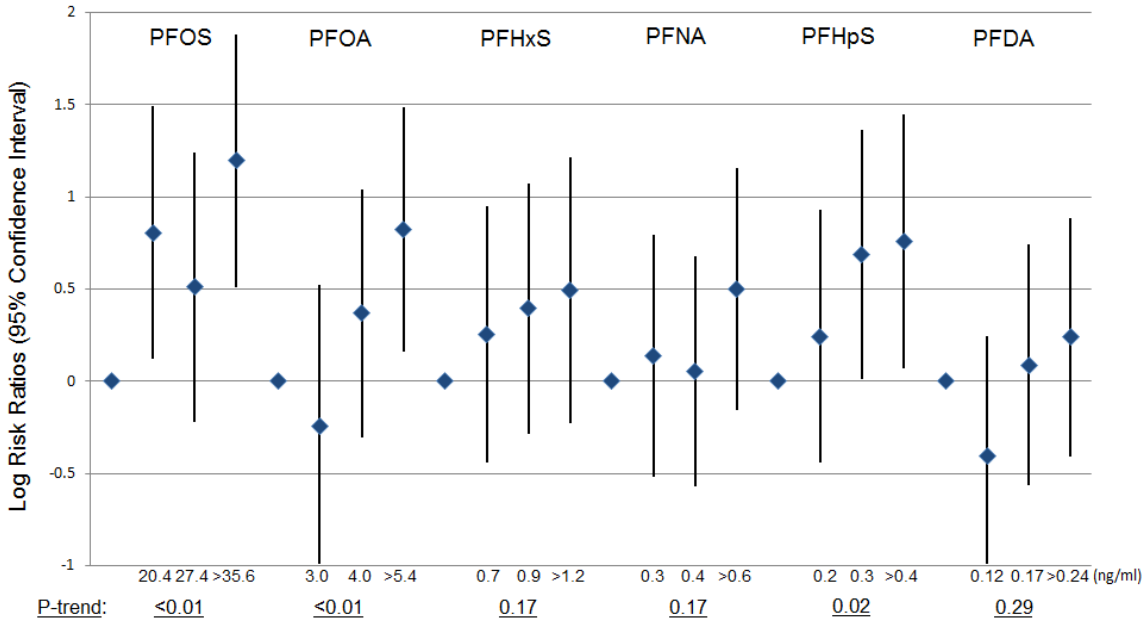


Figure 4.1 Associations of CP in boys and maternal PFAS concentrations (in quartiles) during pregnancy.
X-axis shows the 25th, 50th, and >75th of each PFASs. The lowest quartile was used as the reference group; Y-axis shows the Risk Ratios (in logarithmic scale) adjusting for maternal age at delivery, mother's SES, parity, maternal drinking and smoking during pregnancy, mother's mental health status, gestational week of blood drawn; P-trend was modeled based on the midpoint of each category.

Supplementary Table 4.1 The detection and quantitation limits of PFASs and the plasma concentrations of maternal PFASs in controls

Abbreviation	Chemical Name	Lower limit of detection (ng/ml)	Lower limit of quantitation (LLOQ) (ng/ml)	Percentage >LLOQ in all samples	PFAS concentrations (ng/ml) among controls				
					Minimum	25th Percentile	Median	75th Percentile	Maximum
PFOS	perfluorooctane sulfonate	0.09	0.28	100%	3.85	20.40	27.40	35.60	103.80
PFOA	perfluorooctanoic acid	0.07	0.20	100%	0.57	3.01	4.00	5.42	17.70
PFHxS	perfluorohexan sulfonate	0.03	0.08	98%	<LLOQ	0.68	0.92	1.23	18.16
PFHpS	perfluoroheptane sulfonate	0.04	0.11	96%	<LLOQ	0.21	0.30	0.41	2.04
PFNA	perfluorononanoic acid	0.09	0.27	92%	<LLOQ	0.35	0.43	0.56	1.70
PFDA	perfluorodecanoic acid	0.03	0.09	90%	<LLOQ	0.12	0.17	0.23	1.12
PFUnA	perfluoroundecanoic acid	0.05	0.15	60%	<LLOQ	<LLOQ	0.18	0.27	0.78
PFHpA	perfluoroheptanoic acid	0.02	0.05	46%	<LLOQ	<LLOQ	<LLOQ	0.10	0.75
PFHxA	perfluorohexanoic acid	0.01	0.03	8%	<LLOQ	<LLOQ	<LLOQ	<LLOQ	0.42
PFPeA	perfluoropentanoic acid	0.06	0.19	6%	<LLOQ	<LLOQ	<LLOQ	<LLOQ	1.16
PFOSA	Perfluorooctanesulfonic acid	0.40	1.19	5%	<LLOQ	<LLOQ	<LLOQ	<LLOQ	6.88
PFDS	perfluorodecane sulfonate	0.12	0.37	4%	<LLOQ	<LLOQ	<LLOQ	<LLOQ	4.14
PFBS	perfluorobutane sulfonate	0.02	0.07	3%	<LLOQ	<LLOQ	<LLOQ	<LLOQ	0.20
PFDoA	perfluorododecanoic acid	0.14	0.41	2%	<LLOQ	<LLOQ	<LLOQ	<LLOQ	0.47
PFTeA	perfluorotetradecanoic acid	0.14	0.41	2%	<LLOQ	<LLOQ	<LLOQ	<LLOQ	0.66
PFTrA	perfluorotridecanoic acid	0.14	0.41	2%	<LLOQ	<LLOQ	<LLOQ	<LLOQ	0.52

Supplementary Table S4.2 Pearson correlation coefficients of maternal PFAS concentrations (ng/ml) in controls

	PFOS	PFOA	PFHxS	PFNA	PFHpS	PFDA
PFOS	1.00	0.71	0.27	0.53	0.86	0.53
PFOA		1.00	0.24	0.55	0.73	0.37
PFHxS			1.00	0.30	0.36	0.16
PFNA				1.00	0.62	0.64
PFHpS					1.00	0.51
PFDA						1.00

[a] PFAS values below the lower limit of quantitation were replaced based on multiple imputations

Supplementary Table S4.3 Risk Ratios for subtypes of spastic CP according to maternal PFAS concentrations during pregnancy, boys only

Prenatal exposure	All spastic CP	Unilateral spastic CP	Bilateral spastic CP
	Adjusted RR[a] (95% CI)	Adjusted RR[a] (95% CI)	Adjusted RR[a] (95% CI)
Per 1 natural-log unit (ng/ml) increase			
PFOS	1.8 (1.1, 3.1)	1.4 (0.6, 3.3)	2.2 (1.1, 4.3)
PFOA	2.0 (1.1, 3.6)	2.5 (1.0, 6.3)	1.8 (0.8, 3.8)
PFHxS	1.0 (0.7, 1.4)	1.4 (0.7, 2.8)	0.9 (0.6, 1.3)
PFHpS	1.4 (0.9, 2.2)	1.7 (0.8, 3.4)	1.3 (0.8, 2.2)
PFNA	1.3 (0.7, 2.3)	1.6 (0.6, 4.3)	1.1 (0.6, 2.4)
PFDA	1.2 (0.8, 1.9)	1.2 (0.6, 2.4)	1.3 (0.7, 2.4)

[a] Adjusted for maternal age at delivery, mother's SES, parity, alcohol consumption during pregnancy, smoking during pregnancy, mother's psychiatric illnesses, and gestational week of blood drawn.
[b] 80 spastic CP in boys with 32 unilateral and 48 bilateral manifestations were included in analyses.

Supplementary Table S4.4 Risks Ratios for CP in children according to maternal PFAS concentrations (in quartiles) during pregnancy, by sex

Prenatal exposure [a]	All			Boys only			
	No. Cases / Controls	Adjusted RR[b] (95% CI)	P-trend [e]	No. Cases / Controls	Adjusted RR[c] (95% CI)	P-trend [e]	Adjusted RR[d] (95% CI)
PFOS (ng/ml)							
3.85 - 20.40	34 / 140	1.0 (ref)		15 / 113	1.0 (ref)		1.0 (ref)
20.41 - 27.40	37 / 135	1.1 (0.7, 1.8)		23 / 104	2.2 (1.1, 4.4)		3.1 (1.4, 7.0)
27.41- 35.60	33 / 136	1.1 (0.6, 1.8)		18 / 111	1.7 (0.8, 3.5)		2.1 (0.8, 5.7)
≥ 35.61	48 / 134	1.7 (1.1, 2.8)	0.02	30 / 107	3.3 (1.7, 6.6)	<0.01	4.2 (1.3, 13.3)
PFOA (ng/ml)							
0.57 - 3.01	34 / 137	1.00 (ref)		17 / 113	1.0 (ref)		1.0 (ref)
3.02 - 4.00	32 / 136	0.8 (0.5, 1.3)		14 / 106	0.8 (0.4, 1.7)		0.7 (0.3, 1.6)
4.01 - 5.42	33 / 136	0.8 (0.5, 1.4)		23 / 111	1.5 (0.7, 2.8)		0.9 (0.4, 2.1)
≥ 5.43	53 / 136	1.4 (0.9, 2.3)	0.09	32 / 105	2.3 (1.2, 4.4)	<0.01	1.5 (0.5, 4.3)
PFHxS (ng/ml)							
<LLOQ - 0.68	35 / 141	1.0 (ref)		18 / 113	1.0 (ref)		1.0 (ref)
0.69 - 0.92	41 / 136	1.2 (0.7, 2.0)		22 / 108	1.3 (0.7, 2.6)		1.2 (0.6, 2.5)
0.93 - 1.23	33 / 133	1.2 (0.7, 2.0)		22 / 107	1.5 (0.8, 2.9)		1.2 (0.6, 2.6)
≥ 1.24	43 / 135	1.3 (0.8, 2.2)	0.35	24 / 107	1.6 (0.8, 3.4)	0.17	1.0 (0.4, 2.4)
PFHpS (ng/ml)							
<LLOQ - 0.21	37 / 140	1.0 (ref)		18 / 112	1.0 (ref)		1.0 (ref)
0.22 - 0.30	36 / 141	0.9 (0.5, 1.4)		21 / 113	1.3 (0.6, 2.5)		1.0 (0.4, 2.1)
0.31 - 0.41	35 / 129	1.1 (0.7, 1.8)		21 / 104	2.0 (1.0, 3.9)		0.8 (0.3, 2.2)
≥ 0.42	44 / 135	1.4 (0.8, 2.2)	0.13	26 / 106	2.1 (1.1, 4.3)	0.02	0.7 (0.2, 2.3)
PFNA (ng/ml)							
<LLOQ - 0.35	46 / 149	1.0 (ref)		19 / 113	1.0 (ref)		1.0 (ref)
0.36 - 0.43	33 / 127	0.8 (0.5, 1.2)		18 / 101	1.2 (0.6, 2.2)		0.8 (0.4, 1.7)
0.44 - 0.56	38 / 138	0.8 (0.5, 1.3)		26 / 120	1.1 (0.6, 2.0)		0.8 (0.4, 1.7)
≥ 0.57	35 / 131	0.8 (0.5, 1.2)	0.28	23 / 101	1.7 (0.9, 3.2)	0.17	0.7 (0.3, 1.7)
PFDA (ng/ml)							
<LLOQ - 0.12	42 / 139	1.0 (ref)		22 / 107	1.0 (ref)		1.0 (ref)
0.13 - 0.17	35 / 159	0.7 (0.5, 1.2)		19 / 131	0.7 (0.4, 1.3)		0.5 (0.2, 1.0)
0.18 - 0.23	41 / 121	1.1 (0.7, 1.8)		21 / 95	1.1 (0.6, 2.1)		0.9 (0.4, 2.0)
≥ 0.24	34 / 126	1.0 (0.6, 1.6)	0.71	24 / 102	1.3 (0.7, 2.4)	0.29	1.1 (0.4, 3.0)

[a] PFAS values below the lower limit of quantitation (LLOQ) were grouped in the lowest quartile.
[b] Adjusted for child's sex, maternal age at delivery, mother's SES, parity, alcohol consumption during pregnancy, smoking during pregnancy, mother's psychiatric illnesses, and gestational week of blood drawn.
[c] Adjusted for maternal age at delivery, mother's SES, parity, alcohol consumption during pregnancy, smoking during pregnancy, mother's psychiatric illnesses, and gestational week of blood drawn.
[d] Adjusted for all covariates in a) plus controlling for all PFASs in the same model.
[e] P-trend was modeled based on the median value of each category.

Chapter V: Prenatal Exposure to Perfluoroalkyl Substances, Attention-deficit/ Hyperactivity Disorder and Autism

5.1 Abstract

Perfluoroalkyl substances (PFASs) are persistent pollutants found to be endocrine disruptive and neurotoxic in animals. Positive correlations between PFASs and neurobehavioral problems in children were reported in cross-sectional data, but findings from prospective studies are limited. We investigated whether prenatal exposure to PFASs increases the risks for attention-deficit/hyperactivity disorder (ADHD) or autism in children. Among 83,389 mother-child pairs enrolled in the Danish National Birth Cohort during 1996–2002, we identified 890 ADHD cases and 301 autism cases from the Danish National Hospital Registry and the Danish Psychiatric Central Registry. From this cohort, we randomly selected 220 cases of ADHD and autism each, and we also randomly selected 550 controls frequency matched by child's sex. Sixteen PFASs were measured in maternal plasma collected in early or mid-pregnancy. We used unconditional logistic regressions to estimate odds ratios (OR). Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) were detected in all samples; 4 other PFASs were quantified in ≥ 90% of the samples. Our results suggest no consistent associations between mothers PFAS plasma levels and ADHD or autism (per ln-ng/ml increase in PFOS and ADHD OR=0.80 (95%CI 0.53-1.19) or autism OR=0.86 (95%CI 0.57-1.30), per ln-ng/ml increase in PFOA and ADHD OR=0.91 (95%CI 0.61-1.38) or autism OR=0.93 (95%CI 0.62-1.41)). In models simultaneously adjusting for all PFASs some associations emerged but the estimates were imprecise. We found no evidence to suggest that prenatal PFAS exposure increases the risks for ADHD or autism in children in the Danish National Birth Cohort.

5.2 Introduction

Perfluoroalkyl substances (PFASs) are a group of man-made fluorine-containing compounds with unique properties making materials stain, oil, and water resistant (Buck et al. 2011). PFASs were broadly used in commercial products since the 1950s. Less favorable properties of PFASs are that they are persistent in the environment and in living organisms, and thus are detected in wildlife and in humans throughout the globe (Houde et al. 2006). Human exposure routes include contamination of food from packaging, bioaccumulation in the food chain, and household dust (D'Eon J and Mabury 2011). Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are the two most frequently used PFASs with estimated biological half-lives in humans between 4 to 5 years (Olsen et al. 2007). PFOS and PFOA concentrations in humans were reported to be decreasing in some countries following a drop in production since 2000 (Kato et al. 2011), but exposure to other short-chain compounds such as perfluorobutane sulfonate (PFBS) and perfluorohexane sulfonate (PFHxS) and long-chain perfluorononanoic acid (PFNA) and perfluorodecanoic acid (PFDA) are reported to be increasing (Glynn et al. 2012).

PFASs can cross the placental barrier and expose the fetus during the most vulnerable period of development (Fei et al. 2007b). Experimental data suggest that PFASs may be developmental neurotoxicants that can affect neuronal cell development (Slotkin et al. 2008), alter cognitive function, and reduce habituation and learning ability in mice (Johansson et al. 2008, Johansson et al. 2009, Viberg, Lee and Eriksson 2013). PFASs also have endocrine disruptive properties (Kjeldsen and Bonefeld-Jorgensen 2013) and might interfere with thyroid hormone function (Long et al. 2013, Lau et al. 2003, Lin et al.

2013, Wang et al. 2014) which is essential in regulating fetal brain development (Porterfield 2000).

Attention-deficit/hyperactivity disorder (ADHD) is considered one of the most common neurobehavioral disorders worldwide characterized by inattention, hyperactivity, increased impulsivity and motivational/emotional dysregulation (Polanczyk et al. 2007). Autism is a neurodevelopmental disorder characterized by impairments in communication and reciprocal social interaction, coupled with repetitive behavior (Pickett and London 2005). The incidence of ADHD and autism has increased over the past decades, and it is suggested that the rise is not solely attributable to changes in diagnostic practices or parental awareness (Faraone et al. 2003, Moller, Sorensen and Thomsen 2007, Hertz-Picciotto and Delwiche 2009). The etiologies are not well understood but both environmental and genetic factors, are thought to contribute to ADHD and autism (Millichap 2008, Lyall, Schmidt and Hertz-Picciotto 2014). ADHD and autism disproportionately affect boys (Arnold 1996), and studies suggest that prenatal exposure to endocrine disrupting chemicals may be associated with the occurrence of both diseases (de Cock, Maas and van de Bor 2012).

A limited number of epidemiologic studies have evaluated the potential neurobehavioral or neurocognitive impact of PFASs and findings were inconclusive. Several cross-sectional studies found higher serum levels of some PFASs to be associated with impulsivity, and reported elevated risks of ADHD in children (Gump et al. 2011, Hoffman et al. 2010, Stein and Savitz 2011). Reverse causality, however, is a concern for these survey data that measure PFAS levels in children already diagnosed with ADHD at time of blood draw. Prenatal exposures to PFOS and PFOA were not associated with

behavioral problems in 7-year-old children assessed with the Strengths and Difficulties Questionnaires in the prospective Danish birth cohort (Fei and Olsen 2011b). A study conducted in a community with high long-term exposure to PFOA in contaminated drinking water, reported that higher in-utero PFOA levels were associated with higher Full Scale Intelligence Quotient (IQ) and decreased ADHD characteristics among children aged 6-12 years (Stein et al. 2013). However, prenatal PFOA levels in this study were not measured but estimated based on exposure modeling that might be more prone to exposure misclassification if the modeling assumptions made are incorrect. Thus, additional studies are needed.

We conducted a case-cohort study within the framework of the Danish National Birth Cohort (DNBC) to examine whether prenatal exposure to PFASs increases the risks of developing ADHD and autism, respectively, in children.

5.3 Methods

The DNBC is a nationwide cohort study of pregnancies and health related outcomes in the children (details have been described elsewhere (Olsen et al. 2001)). Briefly, pregnant women were recruited through their general practitioners during early gestation (weeks 6 to 12) from 1996 to 2002. About 50% of all general practitioners in Denmark participated in the study, and 60% of the women invited agreed to participate. Women were ineligible if they did not speak sufficient Danish for interviews or intended not to carry their pregnancy to term. Information was collected in four computer-assisted telephone interviews (twice during pregnancy and twice postpartum). Two prenatal

maternal blood samples were collected and stored, one each in the first and second trimester. English versions of questionnaires are available online at http://www.bsmb.dk.

Source population

The source population for this study consisted of live born singletons, and mothers who participated in the first telephone interview conducted approximately during the 12th gestational week and had provided a blood sample drawn at least once either during the first or second pregnancy trimesters. This resulted in 83,389 mother-child pairs with 42,737 boys and 40,652 girls; we excluded from the original DNBC those with an unsuccessful pregnancy (n=6,207), non-singleton births (n=2,080), births with unknown birth outcomes (n=25) or missing dates of birth (n=99), mothers who emigrated (n=51) or died (n=3), and women who did not participate in the first telephone interview (n=4,578) or did not provide a prenatal blood sample (n=4,609).

Selection of cases and controls

We identified children who were diagnosed with ADHD and autism, respectively, by linking DNBC records to the Danish National Hospital Registry (Andersen et al. 1999) that contains the nationwide data for all admissions for somatic illnesses, and also to the Danish Psychiatric Central Registry (Munk-jorgensen et al. 1993) which covers admissions to all psychiatric hospitals in Denmark. The record linkage relied on the unique civil registration numbers given to all Danish citizens at birth. All diagnoses are based on the International Classification of Diseases, 10th version (ICD10 F90.0 for ADHD; F84.0 for infantile autism) and included inpatients and outpatients records. A total of 890 ADHD cases and 301 autism cases were identified in the cohort during an

average of 10.7 years of follow-up (record linkage was conducted on August 1st, 2011). Due to the high costs of measuring PFASs we randomly selected 220 cases of ADHD and autism each for inclusion in this study.

We randomly selected 550 children (440 males and 110 females) as controls from the source population, frequency matched to cases by sex. The flowchart of subject selection and sampling fractions of cases and controls are shown in Figure 3.1 in Chapter III.

PFAS measurements

Details about analytic methods for PFASs have been described previously (Liew et al. in review). Briefly, the collected maternal blood samples were sent by mail to Statens Serum Institut in Copenhagen, and separated and stored in freezers at -80°C. We used 0.1 ml stored maternal plasma and the samples were analyzed at the Department of Environmental Science at Aarhus University. Solid Phase Extraction (SPE) technique was used for extraction and purification using 0.1 ml maternal plasma; PFAS concentrations were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Measurements were performed in a random sequence for cases and controls by laboratory personnel blinded to diagnoses and any other participant information. Seventeen maternal samples (5 ADHD, 7 autism, 5 controls) were either not available from the biobank or failed the PFAS extraction and purification process hence were excluded. For quality control, we compared the current PFOA and PFOS values with earlier measurements performed at the 3M Toxicology laboratory (Fei et al. 2007b); we found the measures from the two labs to be highly correlated (Pearson correlation r=0.94 for PFOS and r=0.95 for PFOA).

Of the 16 different PFASs detected in maternal plasma, we focus on the 6 PFASs for which at least 90% of all samples were above the lower limit of quantitation (LLOQ): PFOS 100%, PFOA 100%, PFHxS 98%, PFHpS (perfluoroheptane sulfonate) 96%, PFNA 92%, PFDA 90%. The full panel for the LLOQ and distribution of all PFASs was reported in Supplementary Table S4.1 in Chapter IV.

Statistical analysis

We used unconditional logistic regression to estimate odds ratios (OR) and 95% confidence intervals (CI) for prenatal PFAS exposures and ADHD or autism, respectively. In addition, we performed weighted analyses by taking into account the sampling fractions of cases and controls, and we calculated risk ratios (RR) using generalized linear models. PFAS concentrations were first analyzed as continuous variables (per natural-log unit increase). We also categorized PFAS values into quartiles according to the distribution among controls using the lowest quartile as the reference group. Moreover, we fitted generalized additive models with a smoothing function of natural-log PFAS values to examine potential non-linear relations. Five knots were set as the upper limit of number of degrees of freedom, and we compared model fit and visually inspected plots of the smoothed data. We did not find evidence for non-linearity between prenatal PFAS levels and ADHD or autism.

We include the following potential confounders in the final models: maternal age at delivery (≤24, 25-29, 30-34, ≥35 years), parity (1, >1), socio-economic status (SES of low/medium, high; derived from the mother's and father's education and occupation), maternal smoking (never, ≤9 cigarette/day, >9 cigarettes/day) and alcohol drinking (yes,

no) during pregnancy, mother's self-reported psychiatric illnesses (yes, no), gestational week of blood draw (4-8, >8 week), child's birth year (1998-2000, 2001-2003), and the matching factor child's sex. Additionally, other potential confounders such as father's age at child's birth, mother's pre-pregnancy body mass index, and season of conception were evaluated but not included in final models since they changed effect estimates of interest minimally (<1%).

To account for PFASs values below the LLOQ when PFAAs were analyzed as continuous variables, we used multiple imputations (Lubin et al. 2004) with the procedures "PROC MI" in SAS including six PFASs and all covariates in the model. Ten simulated complete datasets were generated via imputation, and we employed standard analytical procedures to combine the results (Yuan 2001).

A Pearson correlation matrix for the considered PFASs is presented in Supplementary Table S4.2 in Chapter IV. We simultaneously included all PFASs in one model to examine whether any single PFAS may be of particular importance. We also evaluated potential effect measure modification by child's sex. In additional sensitivity analyses we excluded PFAS values that were greater than three times the 75th percentile to ensure that individuals with extreme exposure values did not disproportionately influence our results. For ADHD we also conducted analyses in which we excluded children born after the year 2000 because the duration of follow-up may not be long enough to identify children with this diagnosis. Analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

5.4 Results

Table 5.1 presents the demographic characteristics of cases and controls. ADHD and autism diagnoses were more frequent in children born to mothers who smoked during pregnancy or who reported ever having been suffering from a psychiatric illness. Table 5.2 shows the median and inter-quartile range distribution of maternal PFAS concentrations during pregnancy in cases and controls.

We generally found no association between ADHD or autism in children and PFAS levels in maternal plasma (modeled as natural log units) (Table 5.3). Effect estimates were similar and close to null for boys and girls, but since both diagnoses were more prevalent in boys the precision of estimates in girls is low (Supplementary Table S5.1). Results were also comparable when we applied sampling weights, but we gained statistical efficiency resulting in narrower confidence intervals such that some positive as well as negative associations between PFASs and ADHD or autism emerged (Supplementary Table S5.2).

When we categorized PFAS values, children born to mothers in the highest quartile of PFDA were at lower risk of ADHD or autism after adjustment for potential confounders (Table 5.4). However, when all PFASs were simultaneously entered into the model, there was some suggestion that higher PFOA and PFNA levels were associated with an increased risk of ADHD, and some positive associations were observed for PFHxS and autism. The precision of these effect estimates increased when sampling weights were applied (Supplementary Table S5.3).

Additional sensitivity analyses restricting the analyses to children born prior to 2001 (Supplementary Table S5.4), and excluding extreme PFAS values did not change our findings (results not shown).

5.5 DISCUSSION

Overall, our results do not suggest that prenatal exposure to PFASs increases the risks for ADHD or autism in children.

Toxicology studies raised concerns that PFASs are neurotoxic, hormonal disruptive, and can impair fetal brain development (Lau et al. 2003, Johansson et al. 2008, Long et al. 2013). However, some neurotoxic effects in rats were observed at doses several orders of magnitude higher than the PFAS levels found in the U.S. and Danish general population (Butenhoff et al. 2009, Fei et al. 2007b). No prior study has to our knowledge examined childhood autism in relation to prenatal PFAS exposure. Several epidemiologic studies have investigated links between PFASs and hyperactivity or behavioral problems in children but the findings are inconclusive (Fei and Olsen 2011b, Hoffman et al. 2010, Stein and Savitz 2011, Stein et al. 2013). Our results are generally consistent with a previous study based on a subsets of children from the Danish National Birth Cohort that reported a lack of associations between prenatal exposure to PFOA or PFOS and ADHD-like behavioral problems in 7-year-old children measured by parental reports relying on the Strength and Difficulty Questionnaire (Fei and Olsen 2011b).

In our study, since several PFASs are moderately to highly correlated, it is thus difficult to disentangle mixture effects or estimate compound-specific effects. In models in which we mutually adjusted for all PFASs, PFOA and PFNA showed an adverse

(positive) dose response pattern for ADHD, and PFHxS appeared to be associated with slightly increased risks of autism. While this may indicate some mixture specific effects, these effect estimates were imprecise and may simply reflect the aberrant behavior of our model due to multi-collinearity. Interestingly, a recent *in-vitro* assay reported concentration-dependent antagonistic effects of PFAS on androgen receptor transactivity, and when using a mixture of compounds (PFHxS, PFOS, PFOA, PFNA, and PFDA) tested simultaneously, they observed an additive or more than additive antagonistic effect on the androgen receptor function (Kjeldsen and Bonefeld-Jorgensen 2013). Also dose-dependent interference with thyroid hormone function *in vitro* has been previously described (Long et al. 2013). Further experimental studies are needed to determine mechanisms of action for PFAS mixtures on biologic targets that could better inform our population-based studies in terms of the most relevant exposure model to be explored.

It has previously been shown that prenatal exposure to PFASs can increase the incidence of fetal demise and resorption in animal models (Abbott et al. 2007, Lau et al. 2007). In humans, PFASs can interfere with sex and thyroid hormone homeostasis (Lau et al. 2003, Luebker et al. 2005, Lin et al. 2013, Wang et al. 2014) and it has been suggested that they may reduce fecundity (Fei 2009, Buck Louis et al. 2013). It is therefore possible that PFASs exposure at a level that reduces fetal or neonatal survival, especially in high risk fetuses and infants susceptible to neurological disorders such as ADHD and Autism, could appear to have a null or even protective effects on adverse neurobehavioral outcomes in children in observational studies affected by selection bias since only life born children can be followed-up and examined.

There are several strengths in our study. First, the PFASs measures were obtained from maternal plasma samples collected in pregnancy prior to the assessment of the outcomes in the children. Previous studies have shown that PFASs are stable in human serum and measurements obtained from serum or plasma samples gave comparable results (Ehresman et al. 2007). High correlations between maternal and cord blood PFASs measures were also reported suggesting that PFASs in maternal plasma is a valid marker of fetal exposure (Fei et al. 2007b). Furthermore, the maternal PFAS levels in our study are similar to those previously measured during the same time period in the U.S. general population (Calafat et al. 2007a). Study participants were selected from a well-defined nationwide pregnancy cohort with sufficient length of follow-up to assess the outcomes of interest. The outcome measures were clinical diagnoses using standardized diagnostic criteria from both the general and psychiatric hospital registries in Denmark, a country with high quality health care and universal coverage for its population. Diagnoses of infantile autism in the psychiatric registry have previously been shown to have high validity (Lauritsen et al. 2010). Follow-up was conducted through record linkage that did not require subjects' responses, thus minimizing chances for selection bias due to subject's non-response.

Our report also has some limitations. Both ADHD and autism are about 4 times more prevalent in boys, and due to cost limitations we were required to sample no more than 220 cases for each diagnostic group resulting in few female cases (n=41 with ADHD; n=33 with autism). Thus, our subgroup analyses by sex were relatively imprecise for girls resulting in effect estimates with wide confidence intervals. For autism, the cases were limited to children diagnosed with childhood autism. While this is the most severe

disorder of the autism spectrum it constitutes of only a part of autistic spectrum disorders, specifically children with Asperger's syndrome and other pervasive development disorders were not studied. Moreover, we have no data for other endocrine disrupting chemicals, preventing us from evaluating possible correlations and interactions of PFASs with other ubiquitous environmental chemicals with these properties such as polychlorinated biphenyls (PCBs), organophosphates, bisphenol A and phthalates (Polanska, Jurewicz and Hanke 2012, de Cock et al. 2012).

In summary, we found no consistent evidence that prenatal PFAS exposures increase the risks for ADHD or autism in children in the Danish National Birth Cohort. Both weak negative associations as well as some positive associations we observed in subgroup analyses should be further explored. It is recommended that future studies analyze a larger sample, assess the potential mixture effects of exposures to different co-occurring endocrine disruptors, and examine more sensitive indicators such as neuropsychological functioning in children.

Table5.1 Characteristic of study participants (ADHD, Autism and population controls)

Characteristic [a]	ADHD (N=220)		Autism (N=220)		Controls (N=550)	
	n	%	n	%	n	%
Child's sex						
Male	179	81.4	187	85.0	440	80.0
Female	41	18.6	33	15.0	110	20.0
Mother age at delivery (years)						
≤ 24	37	16.8	28	12.7	42	7.6
25-29	83	37.7	81	36.8	235	42.7
30-34	72	32.7	75	34.1	201	36.5
≥ 35	28	12.7	36	16.4	72	13.1
Socio-economic status						
Low / Medium	112	50.9	74	33.6	209	38.0
High	106	48.2	144	65.5	339	61.6
Parity						
1	107	48.6	119	54.1	247	44.9
>1	100	45.5	96	43.6	288	52.4
Maternal drinking during pregnancy						
No	79	35.9	79	35.9	161	29.3
Yes	141	64.1	141	64.1	389	70.7
Maternal smoking during pregnancy						
Never	139	63.2	142	64.5	409	74.4
≤ 9 cigarettes/day	32	14.5	33	15.0	64	11.6
> 9 cigarettes/day	49	22.3	45	20.5	77	14.0
Mother's self-reported psychiatric illnesses						
No	167	75.9	173	78.6	469	85.3
Yes	53	24.1	47	21.4	81	14.7
Child's birth year						
1998-2000	133	60.5	114	51.8	322	58.5
2001-2003	87	39.5	106	48.2	228	41.5
Gestational weeks at blood draw						
4-8 weeks	87	39.5	88	40.0	216	39.3
>8 weeks	119	54.1	115	52.3	305	55.5

[a] The missing values for socio-economic status, parity, and gestational weeks at blood draw are about 1%,4%, 7% respectively.

Table5.2 Distribution of maternal plasma PFAS concentrations in ADHD or Autism cases and controls

Perfluoroalkyl substances	Abbreviation	Carbon chain length[a]	Percentage quantifiable in all samples	PFAS concentrations in ng/ml (median; 25th-75th)[b]		
				ADHD (N=215)	Autism (N=213)	Controls (N=545)
Perfluorooctane sulfonate	PFOS	8	100%	26.80 (19.20-35.00)	25.40 (18.73-32.40)	27.40 (20.40-35.60)
Perfluorooctanoic acid	PFOA	8	100%	4.06 (3.08-5.50)	3.88 (3.08-5.28)	4.00 (3.01-5.42)
Perfluorohexan sulfonate	PFHxS	6	98%	0.84 (0.61-1.15)	0.92 (0.70-1.17)	0.92 (0.68-1.23)
Perfluoroheptane sulfonate	PFHpS	7	96%	0.30 (0.20-0.40)	0.28 (0.19-0.38)	0.30 (0.21-0.41)
Perfluorononanoic acid	PFNA	8	92%	0.42 (0.34-0.52)	0.41 (0.33-0.51)	0.43 (0.35-0.56)
Perfluorodecanoic acid	PFDA	9	89%	0.15 (0.11-0.20)	0.15 (0.11-0.20)	0.17 (0.12-0.23)

[a] The number of carbons in the fully-fluorinated alkyl chain.

[b] Concentrations for 17 samples (5 ADHD, 7 autism and 5 controls) were missing because the samples were either not available from the biobank or failed the extraction process.

Table3 Odds ratios for ADHD and autism in children according to maternal plasma concentrations of PFAS in pregnancy

Prenatal exposure	ADHD[a]			Autism[a]		
	Crude OR	Adjusted OR[b] (95% CI)	Adjusted OR[c] (95% CI)	Crude OR	Adjusted OR[b] (95% CI)	Adjusted OR[c] (95% CI)
Per 1 natural-log unit (ng/ml) increase						
PFOS	0.83	0.80 (0.53-1.19)	0.95 (0.43-2.10)	0.68	0.86 (0.57-1.30)	1.10 (0.50-2.42)
PFOA	0.98	0.91 (0.61-1.38)	1.13 (0.55-2.29)	0.87	0.93 (0.62-1.41)	1.11 (0.54-2.28)
PFHxS	0.93	0.92 (0.72-1.19)	0.98 (0.73-1.32)	1.05	1.09 (0.84-1.41)	1.22 (0.89-1.68)
PFNA	0.75	0.79 (0.53-1.19)	1.09 (0.56-2.11)	0.75	0.79 (0.52-1.19)	0.89 (0.46-1.69)
PFHpS	0.90	0.86 (0.65-1.15)	0.95 (0.54-1.68)	0.80	0.88 (0.67-1.17)	0.85 (0.50-1.44)
PFDA	0.71	0.75 (0.57-0.99)	0.84 (0.55-1.27)	0.74	0.78 (0.57-1.05)	0.84 (0.52-1.35)

[a] 215 ADHD cases, 213 autism cases, and 545 controls were used in analyses.

[b] Adjusted for maternal age at delivery, SES, parity, smoking and drinking during pregnancy, psychiatric illnesses, gestational week of blood drawn, child's sex and birth year.

[c] Adjusted for all covariates in b) additionally including all PFASs in the model.

Table4 Odds ratios for ADHD and autism in children according to maternal plasma concentrations of PFAS (in quartiles) in pregnancy

Prenatal exposure [a]	ADHD				Autism			
	No. Cases / Controls	Crude OR	Adjusted OR[b] (95% CI)	Adjusted OR[c] (95% CI)	No. Cases / Controls	Crude OR	Adjusted OR[b] (95% CI)	Adjusted OR[c] (95% CI)
PFOS (ng/ml)								
3.85 - 20.40	62 / 140	1.00	1.00 (ref)	1.00 (ref)	69 / 140	1.00	1.00 (ref)	1.00 (ref)
20.41 - 27.40	53 / 135	0.92	0.90 (0.57-1.43)	0.89 (0.52-1.51)	51 / 135	0.77	0.87 (0.55-1.37)	0.92 (0.54-1.57)
27.41- 35.60	53 / 136	0.91	0.88 (0.55-1.41)	0.79 (0.40-1.54)	53 / 136	0.79	0.99 (0.62-1.58)	1.15 (0.60-2.23)
≥ 35.61	47 / 134	0.85	0.73 (0.44-1.20)	0.63 (0.28-1.42)	40 / 134	0.61	0.81 (0.48-1.34)	1.05 (0.45-2.45)
PFOA (ng/ml)								
0.57 - 3.01	49 / 137	1.00	1.00 (ref)	1.00 (ref)	53 / 137	1.00	1.00 (ref)	1.00 (ref)
3.02 - 4.00	53 / 136	1.06	1.02 (0.63-1.64)	1.28 (0.74-2.20)	60 / 136	1.14	1.10 (0.70-1.74)	1.13 (0.66-1.92)
4.01 - 5.42	56 / 136	1.11	1.09 (0.67-1.76)	1.54 (0.82-2.87)	55 / 136	1.05	1.04 (0.65-1.68)	1.02 (0.56-1.88)
≥ 5.43	57 / 136	1.12	1.11 (0.66-1.86)	2.02 (0.95-4.27)	45 / 136	0.86	0.92 (0.54-1.54)	0.94 (0.43-2.03)
PFHxS (ng/ml)								
<LLOQ - 0.68	63 / 141	1.00	1.00 (ref)	1.00 (ref)	48 / 141	1.00	1.00 (ref)	1.00 (ref)
0.69 - 0.92	60 / 136	0.99	0.96 (0.61-1.50)	0.85 (0.52-1.39)	59 / 136	1.27	1.33 (0.83-2.11)	1.44 (0.86-2.39)
0.93 - 1.23	53 / 133	0.92	0.86 (0.54-1.36)	0.75 (0.44-1.29)	62 / 133	1.37	1.47 (0.92-2.35)	1.80 (1.04-3.10)
≥ 1.24	39 / 135	0.73	0.60 (0.36-1.00)	0.54 (0.29-1.01)	44 / 135	0.96	1.03 (0.62-1.72)	1.30 (0.70-2.43)
PFNA (ng/ml)								
<LLOQ - 0.35	62 / 149	1.00	1.00 (ref)	1.00 (ref)	66 / 149	1.00	1.00 (ref)	1.00 (ref)
0.36 - 0.43	53 / 127	1.00	1.05 (0.67-1.66)	1.23 (0.75-2.02)	56 / 127	1.00	1.08 (0.69-1.68)	0.99 (0.60-1.62)
0.43 - 0.56	60 / 138	1.03	1.15 (0.74-1.78)	1.57 (0.91-2.70)	51 / 138	0.83	0.82 (0.52-1.28)	0.76 (0.43-1.32)
≥ 0.57	40 / 131	0.80	0.82 (0.50-1.33)	1.52 (0.77-3.03)	40 / 131	0.69	0.78 (0.49-1.26)	0.88 (0.43-1.80)
PFHpS (ng/ml)								
<LLOQ - 0.21	65 / 140	1.00	1.00 (ref)	1.00 (ref)	66 / 140	1.00	1.00 (ref)	1.00 (ref)
0.21 - 0.30	48 / 141	0.80	0.71 (0.45-1.12)	0.70 (0.40-1.21)	55 / 141	0.83	0.83 (0.53-1.30)	0.76 (0.45-1.30)
0.30 - 0.41	54 / 129	0.93	0.84 (0.53-1.34)	0.87 (0.44-1.73)	49 / 129	0.81	0.90 (0.56-1.43)	0.83 (0.42-1.61)
≥ 0.42	48 / 135	0.83	0.67 (0.41-1.11)	0.82 (0.34-1.99)	43 / 135	0.68	0.80 (0.49-1.32)	0.84 (0.35-2.03)
PFDA (ng/ml)								
<LLOQ - 0.12	70 / 139	1.00	1.00 (ref)	1.00 (ref)	64 / 139	1.00	1.00 (ref)	1.00 (ref)
0.13 - 0.17	66 / 159	0.88	0.77 (0.50-1.18)	0.79 (0.50-1.26)	69 / 159	0.94	0.92 (0.60-1.41)	1.00 (0.64-1.58)
0.18 - 0.23	49 / 121	0.86	0.93 (0.58-1.48)	1.01 (0.58-1.78)	53 / 121	0.95	1.08 (0.68-1.71)	1.40 (0.80-2.45)
≥ 0.24	30 / 126	0.57	0.52 (0.31-0.87)	0.59 (0.29-1.18)	27 / 126	0.47	0.52 (0.30-0.88)	0.77 (0.37-1.60)

[a] PFAS values below the lower limit of quantitation (LLOQ) were grouped in the lowest quartile.
[b] Adjusted for maternal age at delivery, SES, parity, smoking and drinking during pregnancy, psychiatric illnesses, gestational week of blood drawn, child's sex and birth year.
[c] Adjusted for all covariates in b) additionally including all PFASs in the model.

Supplementary Table S5.1 Odds ratios for ADHD and autism in boys and girls according to maternal plasma concentrations of PFAS in pregnancy

Prenatal exposure	ADHD[a]		Autism[a]	
	Boys only	**Girls only**	**Boys only**	**Girls only**
	Adjusted OR[b] (95% CI)	Adjusted OR[b] (95% CI)	Adjusted OR[b] (95% CI)	Adjusted OR[b] (95% CI)
Per 1 natural-log unit (ng/ml) increase				
PFOS	0.81 (0.52-1.27)	0.92 (0.30-2.82)	0.99 (0.63-1.55)	0.53 (0.15-1.86)
PFOA	0.92 (0.58-1.45)	1.13 (0.37-3.48)	1.07 (0.68-1.68)	0.52 (0.16-1.76)
PFHxS	0.94 (0.72-1.23)	0.97 (0.38-2.45)	1.16 (0.87-1.54)	0.89 (0.34-2.35)
PFNA	0.76 (0.47-1.22)	1.15 (0.44-2.98)	0.85 (0.54-1.33)	0.62 (0.22-1.74)
PFHpS	0.86 (0.63-1.18)	0.98 (0.42-2.29)	0.97 (0.71-1.33)	0.72 (0.31-1.69)
PFDA	0.70 (0.51-0.96)	1.01 (0.46-2.24)	0.85 (0.60-1.20)	0.52 (0.23-1.18)

[a] 215 ADHD cases (176 boys, 39 girls), 213 autism cases (180 boys, 33 girls) and 545 controls (435 boys and 110 girls) were used in analyses.

[b] Adjusted for maternal age at delivery, SES, parity, smoking and drinking during pregnancy, psychiatric illnesses, gestational week of blood drawn, and birth year.

Supplementary Table S5.2 Risks ratios for ADHD and autism in children according to maternal plasma concentrations of PFAS in pregnancy

	ADHD[a]		Autism[a]	
Prenatal exposure	Adjusted RR[b] (95% CI)	Adjusted RR[c] (95% CI)	Adjusted RR[b] (95% CI)	Adjusted RR[c] (95% CI)
Per 1 natural-log unit (ng/ml) increase				
PFOS	0.87 (0.74-1.02)	1.04 (0.70-1.56)	0.92 (0.69-1.22)	1.21 (0.69-2.13)
PFOA	0.98 (0.82-1.16)	1.21 (0.84-1.74)	0.98 (0.73-1.31)	1.15 (0.68-1.93)
PFHxS	0.97 (0.88-1.08)	1.05 (0.91-1.20)	1.10 (0.92-1.33)	1.26 (1.00-1.58)
PFNA	0.80 (0.62-1.03)	0.99 (0.58-1.70)	0.80 (0.58-1.11)	0.84 (0.48-1.49)
PFHpS	0.91 (0.79-1.05)	0.93 (0.64-1.36)	0.91 (0.74-1.12)	0.82 (0.56-1.22)
PFDA	0.76 (0.64-0.91)	0.80 (0.58-1.11)	0.79 (0.63-1.01)	0.82 (0.53-1.28)

[a] 215 ADHD cases, 213 autism cases, and 545 controls were used in analyses.
[b] Adjusted for maternal age at delivery, SES, parity, smoking and drinking during pregnancy, psychiatric illnesses, gestational week of blood drawn, child's sex and birth year.
[c] Adjusted for all covariates in b) additionally including all PFASs in the model.
[d] Inverse probability weights derived from sampling fractions of cases and controls were applied in analyses.

Supplementary Table S5.3 Risks ratios for ADHD and autism in children according to maternal plasma concentrations of PFAS (in quartiles) in pregnancy

	ADHD			Autism		
Prenatal exposure[a]	Crude RR	Adjusted RR[b] (95% CI)	Adjusted RR[c] (95% CI)	Crude RR	Adjusted RR[b] (95% CI)	Adjusted RR[c] (95% CI)
PFOS (ng/ml)						
3.85 - 20.40	1.00	1.00 (ref)	1.00 (ref)	1.00	1.00 (ref)	1.00 (ref)
20.41 - 27.40	0.83	0.95 (0.79-1.15)	0.93 (0.75-1.15)	0.72	0.91 (0.66-1.25)	1.05 (0.73-1.50)
27.41- 35.60	0.90	0.93 (0.76-1.13)	0.86 (0.65-1.12)	0.80	1.01 (0.73-1.40)	1.20 (0.77-1.89)
≥ 35.61	0.78	0.79 (0.64-0.98)	0.65 (0.47-0.91)	0.60	0.86 (0.59-1.25)	1.16 (0.65-2.09)
PFOA (ng/ml)						
0.57 - 3.01	1.00	1.00 (ref)	1.00 (ref)	1.00	1.00 (ref)	1.00 (ref)
3.02 - 4.00	1.00	1.02 (0.84-1.23)	1.24 (0.99-1.55)	1.05	1.13 (0.82-1.56)	1.11 (0.76-1.60)
4.01 - 5.42	1.13	1.09 (0.90-1.33)	1.46 (1.14-1.88)	1.03	1.05 (0.74-1.47)	0.97 (0.63-1.48)
≥ 5.43	1.07	1.14 (0.92-1.40)	2.02 (1.49-2.75)	0.78	0.95 (0.65-1.38)	0.93 (0.54-1.59)
PFHxS (ng/ml)						
<LLOQ - 0.68	1.00	1.00 (ref)	1.00 (ref)	1.00	1.00 (ref)	1.00 (ref)
0.69 - 0.92	0.97	1.05 (0.88-1.26)	0.94 (0.76-1.15)	1.26	1.33 (0.95-1.87)	1.55 (1.06-2.28)
0.93 - 1.23	0.90	0.94 (0.78-1.14)	0.82 (0.65-1.02)	1.38	1.50 (1.08-2.10)	1.86 (1.25-2.76)
≥ 1.24	0.64	0.67 (0.54-0.83)	0.56 (0.43-0.73)	0.94	1.07 (0.73-1.56)	1.33 (0.84-2.11)
PFNA (ng/ml)						
<LLOQ - 0.35	1.00	1.00 (ref)	1.00 (ref)	1.00	1.00 (ref)	1.00 (ref)
0.36 - 0.43	1.07	1.08 (0.90-1.30)	1.29 (1.05-1.59)	1.06	1.06 (0.78-1.44)	0.94 (0.66-1.34)
0.43 - 0.56	1.28	1.12 (0.93-1.33)	1.48 (1.18-1.86)	1.03	0.81 (0.59-1.11)	0.73 (0.49-1.08)
≥ 0.57	0.75	0.85 (0.69-1.04)	1.58 (1.17-2.13)	0.70	0.80 (0.56-1.12)	0.98 (0.59-1.63)
PFHpS (ng/ml)						
<LLOQ - 0.21	1.00	1.00 (ref)	1.00 (ref)	1.00	1.00 (ref)	1.00 (ref)
0.21 - 0.30	0.74	0.70 (0.58-0.84)	0.67 (0.54-0.83)	0.83	0.82 (0.60-1.12)	0.70 (0.49-1.01)
0.30 - 0.41	0.91	0.87 (0.72-1.05)	0.86 (0.65-1.13)	0.82	0.92 (0.66-1.29)	0.83 (0.53-1.31)
≥ 0.42	0.75	0.71 (0.58-0.87)	0.81 (0.57-1.15)	0.66	0.82 (0.57-1.19)	0.80 (0.44-1.48)
PFDA (ng/ml)						
<LLOQ - 0.12	1.00	1.00 (ref)	1.00 (ref)	1.00	1.00 (ref)	1.00 (ref)
0.13 - 0.17	0.91	0.82 (0.69-0.97)	0.80 (0.66-0.96)	1.04	0.93 (0.69-1.25)	0.99 (0.72-1.37)
0.18 - 0.23	0.83	0.87 (0.72-1.05)	0.91 (0.73-1.14)	0.98	1.07 (0.77-1.47)	1.34 (0.92-1.95)
≥ 0.24	0.51	0.53 (0.43-0.66)	0.53 (0.40-0.72)	0.50	0.52 (0.35-0.77)	0.73 (0.43-1.24)

[a] PFAS values below the lower limit of quantitation (LLOQ) were grouped in the lowest quartile.
[b] Adjusted for maternal age at delivery, SES, parity, smoking and drinking during pregnancy, psychiatric illnesses, gestational week of blood drawn, child's sex and birth year.
[c] Adjusted for all covariates in b) additionally including all PFASs in the model.
[d] Inverse probability weights derived from sampling fractions of cases and controls were applied in analyses.

Supplementary Table S5.4 Odds ratios for ADHD and autism according to maternal plasma concentrations of PFAS in pregnancy, among children born 1998-2000

	ADHD[a]		Autism[b]	
Prenatal exposure	Adjusted OR[b] (95% CI)	Adjusted OR[c] (95% CI)	Adjusted OR[b] (95% CI)	Adjusted OR[c] (95% CI)
Per 1 natural-log unit (ng/ml) increase				
PFOS	0.62 (0.36-1.06)	0.71 (0.22-2.29)	0.77 (0.42-1.39)	1.53 (0.43-5.40)
PFOA	0.95 (0.54-1.66)	1.74 (0.68-4.44)	0.82 (0.45-1.50)	1.03 (0.37-2.90)
PFHxS	0.92 (0.68-1.23)	1.02 (0.72-1.44)	1.00 (0.72-1.38)	1.16 (0.77-1.74)
PFNA	0.65 (0.37-1.12)	0.96 (0.44-2.10)	0.75 (0.41-1.36)	0.96 (0.39-2.37)
PFHpS	0.69 (0.45-1.07)	0.82 (0.31-2.14)	0.73 (0.47-1.14)	0.60 (0.22-1.64)
PFDA	0.62 (0.42-0.90)	0.77 (0.44-1.33)	0.70 (0.43-1.13)	0.84 (0.34-2.04)

[a] 129 ADHD cases, 109 autism cases and 317 controls were used in analyses.
[b] Adjusted for maternal age at delivery, SES, parity, smoking and drinking during pregnancy, mother's psychiatric illnesses, gestational week of blood drawn, child's sex, and child's birth year.
[c] Adjusted for all covariates in b) additionally including all PFASs in the model.

Chapter VI: Does conditioning on live-birth have the capacity to induce bias in birth cohort studies?

6.1 Abstract

Human reproduction involves a complex selection process and the "right denominators" - the time at risks for fetuses starting from conception onward is often missing. In epidemiology, health outcomes that can only be ascertained in life born children will be missing for all death and aborted fetuses. Thus, for these outcomes, assessment of all pregnancy exposure and event associations necessarily condition on live birth status. We used simulation techniques and 'priors' taken from the Danish National Birth Cohort to investigate whether conditioning on live-born status may induce sufficiently large bias to explain an unexpected inverse associations between prenatal exposure to Perfluoroalkyl substances (PFAS) and ADHD in our and previous studies. In this simulation study, we found that some protective associations for PFAS on ADHD observed in the live-born cohort could possibly be explained by selection bias if PFAS reduces fetal survivals. The magnitude of this selection bias due to fetal death is generally small and depends on whether the exposure is a strong determinant of fetal loss and if there are uncontrolled risk factors of the outcome that also reduce chances of fetal survival. For this mechanism we proposed, one way to reduce or even eliminate the bias due to conditioning on fetal survival is to adjust for the common causes of the outcome and fetal losses in analyses. This highlights the needs for a better understanding of the determinants of pregnancy loss and these important data should be collected in birth cohort studies.

6.2 Introduction

In life-course reproductive epidemiology, we often aim to study exposure induced fetal programming as a function of the time the population at risk (fetus) spent under observation and this time starts at conception. However, the total number of conceptions in the source population is generally unknown because of complex selection phenomena in human reproduction such that only 60-70% of fertilized eggs will results in a live birth (Wilcox et al. 1988, Chard 1991). Early fetus loss usually goes unnoticed and undocumented. Outcomes that can only be ascertained in life born children will be missing all dead and aborted fetuses that never were born alive. Therefore, for childhood outcomes as a consequence of pregnancy exposures, exposure event relationships necessarily condition on live birth status, yet few studies have quantitatively examined the impact this may have on study results.

A few studies have attempted to estimate the incidence of early and total pregnancy loss. An early study following 221 healthy women attempting to conceive relied on the rise of human chorionic gonadotropin (hCG) to detect early pregnancy, and the study reported a ~22% early pregnancy loss (ELP; defined as fetal lost after implantation and before pregnancy was detected clinically), and a ~31% total pregnancy loss including clinically recognized abortions (Wilcox et al. 1988). Later studies collaborated these findings: a US study estimated ELP in the range from 11-27% (Ellish et al. 1996), and the Danish first-pregnancy planner study reported ELP as 12-22% (Bonde et al. 1998). Using hCG as a biomarkers however does not allow for pregnancy loss detection before implantations, and the rate of EPL is expected to be higher when adding the loss prior to implantations (Macklon, Geraedts and Fauser 2002, Chard 1991).

Approximately 60% of all spontaneous abortions are thought to be related to genetic, infectious, hormonal, and/or immunological factors (Philipp and Kalousek 2002, Giacomucci et al. 1994). In addition, exposure to environmental chemicals such as endocrine disrupting compounds (Weselak et al. 2008), heavy metals (Ajayi, Charles-Davies and Arinola 2012), pesticides(Arbuckle and Sever 1998), and cigarette smoke (Kline et al. 1977) were suggested to affect reproduction contributing to infertility and pregnancy loss in human.

Perfluoroalkyl substances (PFASs) are man-made chemicals that are being extensively used in industrial and house-hold products. PFASs are persistent and bioaccumulative and exposure is widespread in the environment and humans. PFASs are able to cross the placental barrier exposing the fetuses during early development (Fei et al. 2007b) and may increase risks of fetal and neonatal deaths (Yahia et al. 2008, Lau et al. 2003). Prenatal exposures to PFASs were suggested to increase incidence of fetal resorptions and pregnancy loss in animals (Case, York and Christian 2001, Lau et al. 2006). In human, PFASs were shown to interfere with sex hormone and thyroid hormone homeostasis (Lau et al. 2003, Luebker et al. 2005, Wen et al. 2013, Lin et al. 2013) and were found to be associated with reduced fecundity (Fei 2009, Buck Louis et al. 2013). In addition, PFASs were reported to have an impact on sperm morphology, semen quality and reproductive hormone levels in men (Joensen et al. 2009, Joensen et al. 2013, Toft et al. 2012) that may also result in reduction of fecundity and an elevated risk for EPL.

Animal studies also showed that PFASs have developmental neurotoxic effects

(Johansson et al. 2008, Johansson et al. 2009, Onishchenko et al. 2011, Viberg et al. 2013), but mixed results were reported by epidemiologic research. Two cross-sectional studies in the US found that PFAS levels correlated with higher risk of attention-deficit/hyperactivity disorder (ADHD) in children (Hoffman et al. 2010, Stein and Savitz 2011). ADHD is one of the most common neurobehavioral disorders with rapidly rising incidence worldwide (Polanczyk et al. 2007). However, two previous reports based on subsets of the Danish National Birth Cohort (DNBC) unexpectedly found some weak inverse associations between prenatal PFASs levels and behavioral problems (Fei and Olsen 2011a) or children clinically diagnosed with ADHD (Liew et al. submitted). Another longitudinal study conducted in a community highly exposed to PFOA through contaminated drinking water for decades contrary to the cross sectional studies reported in-utero exposure to perfluorooctanoate (PFOA) to be associated with fewer not more ADHD symptoms (Stein et al. 2013). There is however no biologic explanation for a protective effect PFASs may have on the developing brain that could explain lower ADHD rates. Chance error is a possible explanation as long as few data are available on this subject, but it is also important to explore potential biases previous studies might have been suffering from.

Here, we use simulation techniques and 'priors' taken from the Danish National Birth Cohort to investigate whether conditioning on live-born status may induce sufficiently large bias to explain unexpected inverse associations between prenatal PFAS exposures and ADHD in our and previous studies.

6.3 Methods

Causal Assumptions

We use directed acyclic graphs (DAGs) to present our causal assumptions and the structural relationship between prenatal exposure to PFASs and ADHD in children. The basic set of rules in utilizing DAGs to express causal relationships for observational studies has been described extensively elsewhere ((Pearl 1995, Greenland, Pearl and Robins 1999)). Two major sources of biasing paths are uncontrolled confounding (Figure 6.1a), i.e. the failure to control for a confounder (or common cause), and conditioning on a collider (common effect of two variables), i.e. opening up an otherwise closed path (Figure 6.1b). A well-known example of collider bias is selection bias resulting from study participants' differential (in terms of outcome and exposure) non-response or loss-to-follow-up (Hernan, Hernandez-Diaz and Robins 2004). Competing risk and survivor bias have also been previously conceptualized as a common form of collider bias (Thompson, Zhang and Arah 2013).

Hypothesized scenarios

Three scenarios were proposed based on priors we considered realistic given prior knowledge regarding PFAS, ADHD, fetal survivals, and known/measured as well as unknown/unmeasured risk factors of ADHD. In the first scenario (Figure 6.2a), there is no causal relationship between PFAS and ADHD. However, exposure to PFAS decreases the chance of conception (C) and fetal survival before clinical detection of pregnancy (S1) or a live birth (S2). Based on the literatures, we introduce a variable R which represents a set of known risk factors for ADHD that also influence conception and fetal survival,

specifically maternal socio-economic status (Russell et al. 2013, Rodriguez et al. 2009), smoking (Langley et al. 2012, Mick et al. 2002), drinking (Torvik et al. 2011, Mick et al. 2002), and psychological stress during pregnancy (Li et al. 2010, Grizenko et al. 2012). In addition, the variable U represents a set of unmeasured or unknown risk factors for ADHD that reduce fecundity and fetal survival. Some possible candidates for U include predisposition due to genetic factors and co-morbidities in neurological diseases (Steinhausen 2009, Abdallah et al. 2011, Larsson et al. 2013, Vorstman and Ophoff 2013). In the second scenario (Figure 6.2b), the same set of variables was employed as in scenario1 but here we now hypothesize that prenatal exposure to PFAS causes ADHD in children.

In addition, we also tested a third scenario (Figure 6.3) where no causal relationship between PFAS and ADHD was assumed, and three unknown or unmeasured risks factor of U were simulated (U1, U2, and U3). U1 represents some unknown predisposition genetic factors that are strong risk factors of ADHD and have a moderate effect on fetal losses. U2 represents some other unmeasured environmental and lifestyle factors that weakly to moderately associated with ADHD and fetal death, and also correlate with PFAS exposures. U3 represent potential unmeasured confounding factors associated with both disease and outcome.

Simulations and statistical analysis

We used Monte-Carlo techniques to perform simulations based on prior knowledge from the Danish National Birth Cohort (DNBC).The DNBC is a nationwide cohort study that followed about 100,000 pregnancies and children with the aim to study pregnancy

complications and diseases in offspring with causes operating during pregnancy and early life (for details see Olsen et al.(Olsen et al. 2001)). Pregnant women were recruited by general practitioners after their pregnancies were clinically recognized around 6–12 weeks of gestation. ADHD diagnoses in children can be ascertained based on ICD-10 diagnoses through linkage with admission records from all general and mental health hospitals in Denmark (Andersen et al. 1999, Munk-jorgensen et al. 1993). Maternal PFAS levels during pregnancy in the DNBC have previously been ascertained and reported (Fei et al. 2007, Liew et al. submitted). Perfluorooctane sulfonate (PFOS) and Perfluorooctanote (PFOA) are the most frequently detected PFASs i.e. they were detected in all maternal plasma samples measured from the DNBC; a few other types of PFASs also were detected in some but not all of the women (Liew et al. submitted).

We simulated a cohort that consisted of about 92,000 live-born singletons matching the numbers in the DNBC with ~3% of all children having developed ADHD. We assumed that 20% of ELP remained undocumented, and that 5% of fetal deaths occurred after cohort enrollment as documented in previous studies reporting on miscarriages and abortions in the DNBC (Norsker et al. 2012, Howards et al. 2012). We compared women with PFASs level in the highest quartile to in the lower 3 quartiles, thus generating a binary PFAS variable with the prevalence of exposure set as 25%. In all three scenarios, fix priors were used for the prevalence of known risk factors (R), and the relationship of R and ADHD or fetal losses. In addition, a range of input levels were assigned to the unknown or unmeasured risks factors (Ux; represents multiple U), including the prevalence, and their relationships with ADHD and fetal losses. The list of variables with

the assumed priors for the prevalence and the strength of associations are presented in Table 6.1.

We use SAS 9.3 (SAS Institute Inc., Cary, NC, USA) to perform simulation and analyses, and the simulation technique using SAS has been described elsewhere (Wicklin 2013). We generated binary values for the 'exogenous' variables (i.e. variables that have no arrow pointing towards them as shown in DAGs in figure2 and figure3) including PFAS in the first two scenario, and the known or unknown risk factors of ADHD (R and Ux) in all scenarios, by random draws from independent Bernoulli distributions such that: PFAS ~ B(1, 0.25), R ~ B(1, 0.4)) and Ux ~ B(1, P(Ux=1)). For variables that have causal determinants (arrows that point towards them) such as conception (C), ADHD and PFAS in the third scenario, simulation was conducted based on following equations:

$C \sim B\ (1, (1/\ (1+ \exp\ (-\ (\log(P(C=1)/1-P(C=1)) + \log(OR_{PFAS-C})*PFAS + \log(OR_{R-C})*R + \log(OR_{U-C})*U)))))$

in senario1:

$ADHD \sim B\ (1, (1/\ (1+ \exp\ (-\ (\log(P(ADHD=1)/1-P(ADHD=1)) + \log(OR_{R-ADHD})*R + \log(OR_{U-ADHD})*U))))$

in senario2:

$ADHD \sim B\ (1, (1/\ 1+ \exp\ (-\ (\log(P(ADHD=1)/1-P(ADHD=1)) + \log(OR_{R-ADHD})*R + \log(OR_{U-ADHD})*U + \log(OR_{PFAS-ADHD})*PFAS))))$

in senario3:

$ADHD \sim B\ (1, (1/\ 1+ \exp\ (-\ (\log(P(ADHD=1)/1-P(ADHD=1)) + \log(OR_{R-ADHD})*R + \log(OR_{U1-ADHD})*U1 + \log(OR_{U2-ADHD})*U2 + \log(OR_{U3-ADHD})*U3))))$

PFAS ~ B (1, (1/ 1+ exp (- (log(P(PFAS=1)/1-P(PFAS=1)) + log($OR_{R\text{-}PFAS}$)*R + log($OR_{U2\text{-}PFAS}$)*U2 + log($OR_{U3\text{-}PFAS}$)*U3))))

For fetal survivals in early pregnancy (S1) and a live born infant (S2), we used a conditional probability such as:

In scenario 1 and 2:

S1 ~ B (1, (1/ (1+ exp (- (log(P(S1=1)/1-P(S1=1)) + log($OR_{PFAS\text{-}S1}$)*PFAS + log($OR_{R\text{-}S1}$)*R + log($OR_{U\text{-}S1}$)*U)))) | concept=1
S2 ~ B (1, (1/ (1+ exp (- (log(P(S2=1)/1-P(S2=1)) + log($OR_{PFAS\text{-}S2}$)*PFAS + log($OR_{R\text{-}S2}$)*R + log($OR_{U\text{-}S2}$)*U)))) | S1=1

In scenario 3:

S1 ~ B (1, (1/ (1+ exp (- (log(P(S1=1)/1-P(S1=1)) + log($OR_{PFAS\text{-}S1}$)*PFAS + log($OR_{R\text{-}S1}$)*R + log($OR_{U1\text{-}S1}$)*U1 + log($OR_{U2\text{-}S1}$)*U2)))) | concept=1
S2 ~ B (1, (1/ (1+ exp (- (log(P(S2=1)/1-P(S2=1)) + log($OR_{PFAS\text{-}S2}$)*PFAS + log($OR_{R\text{-}S2}$)*R + log($OR_{U1\text{-}S1}$)*U1 + log($OR_{U2\text{-}S1}$)*U2)))) | S1=1

For each simulated dataset, we performed logistic regression of ADHD status in children on prenatal PFAS exposure, restricting to live births only (S2=1), with further assumptions that all children survived after birth and there was no loss-to-follow-up preventing us from knowing the outcome status. The analysis was repeated for each level of the different priors as inputs in each scenario. We reported Odds ratios and 95% simulation intervals using the 2.5, 50, and 97.5 percentile under the 1000 simulation attempts. We compared the estimates with or without adjustment for known common causes of ADHD and fetal death (R) in scenario 1 and 2.

6.4 Results

In senario1 where a true null association among PFAS and ADHD was assumed, we observed a protective effect of PFAS on ADHD among live-births after conditioning on fetal survivals (Table 2). As expected, the magnitude of the inverse associations was larger when PFASs were assumed to have stronger impacts on conception, early and late pregnancy loss. Moreover, the estimated protective associations became stronger when the effect sizes for unmeasured risk factors for ADHD and pregnancy loss were increased. The largest protective effects estimated were ORcrude=0.77 (95%CI 0.70-0.83) and ORadjusted= 0.81 (95%CI 0.74-0.89). All adjusted ORs were closer to the null than crude ORs, suggesting that controlling for the known risks factors for ADHD and fetal deaths in the analyses attenuates the negative bias slightly. Increasing the prevalence of the unknown factors changed the estimated bias minimally.

We assumed causal relationships of moderate size magnitude between PFAS and ADHD in scenario 2 (true OR=1.20 or 1.50). When the true OR of prenatal exposure to PFAS and ADHD were assumed to be 1.20, a bias resulting towards the null was found for most results when conditioning on fetal survival, but only a few estimates crossed null and showed OR below 1 (Table 3). An attenuated positive association was also seen for stronger assumed effect of PFAS on ADHD (true OR=1.50), but none of the point estimates fell below one (Table 4). Again, adjusting for known common causes of ADHD and fetal death removed some of the negative biases and raised the effect estimates to be closer to the simulated true OR.

Table 5 shows the results in scenario 3 (true OR = 1.00 for PFAS and ADHD) where uncontrolled confounding was also assumed in addition to selection bias due to fetal death. The observed association of PFAS and ADHD appeared to be either positive or negative in this scenario, and largely depended on the direction and magnitude of the uncontrolled confounding effect; the strongest inverse-association between PFAS and ADHD was found when PFAS has a strong impact on fetal loss and there was an uncontrolled negative confounding bias.

6.5 Discussion

In this study we simulated a realistic pregnancy cohort based on the distributions of factors observed in the DNBC and prior knowledge about associations. We found that weak protective associations of PFAS on ADHD observed in a cohort with live-born children only could be explained by selection bias if PFAS reduces fetal survivals. The magnitude of this negative bias depends on the strengths of associations between PFAS and fetal deaths and between uncontrolled risks factors for ADHD that are also correlated with fetal deaths. In different hypothesized scenarios in this birth cohort, we found that PFAS may appear to be protective as long as it has a true null effect on ADHD while a true positive effect would be attenuated towards the null but would be unlikely to produce an inverse association. Adjusting for common causes of ADHD and fetal deaths could partially or entirely remove the negative bias. Furthermore, uncontrolled confounding in the scenarios could contribute either additional positive or negative biases to the PFAS and ADHD associations.

The etiology of ADHD is not well understood but both environmental and genetic factors are suggested to contribute (Millichap 2008, Halmoy et al. 2012). Several cross-sectional studies have previously reported a positive correlation between current level of PFAS and ADHD in children (Stein and Savitz 2011, Hoffman et al. 2010), however two recent longitudinal studies found some unexpected inverse-association between prenatal PFAS levels and ADHD risks in children through follow-up (Stein et al. 2013)(Liew et al. submitted). Chance errors might explain the negative correlations between prenatal PFAS and ADHD since the studies thus far relied on a relatively small number of cases. Previous studies also mentioned that PFOA have been shown to activate human in vitro peroxisome proliferator-activated receptor (PPAR) alpha and gamma, and given that PPAR-gamma agonists are reportedly neuroprotective and have anti-inflammatory properties suggested that these effects of PFAO might even be beneficial (Power et al. 2013, Stein et al. 2013). Yet, this explanation contradicts the toxicologic neurotoxic effects of PFASs in experimental models. Our simulation study provided an alternative explanation where PFASs having an impact on fetal losses would lead to bias that suggests protective association for prenatal PFAS and ADHD.

Some cautions should be taken in the interpretations of our study. The results are merely meant to be illustrative where we use PFAS and ADHD as an example to show the magnitude and direction of possible biases induced by conditioning on live-born status in observational researches if the assumptions we made for modeling hold. We presented several underlying causal structures and conducted our simulations according to the DAGs and the priors we showed. Although prenatal exposure to PFASs was shown to cause fetal and neonatal deaths in animals (Zheng et al. 2011, Yahia et al. 2010), it is still

debated whether PFASs may have an impact on conception or fetal loss in human (Kristensen et al. 2013, Fei 2009, Whitworth et al. 2012, Vestergaard et al. 2012, Buck Louis et al. 2013). Moreover, it is in general reasonable to believe that there are uncontrolled risk factors of ADHD but knowledge of whether or how strong these factors are correlated with fertility and fetal loss are limited; we thus varied the strength of the assumed associations for all unknown factors and presented multiple scenarios for our simulations. The estimates in our study should also not be directly compared to previous reports based on actual data and modeling in the DNBC (Liew et al submitted), because we used much simpler scenarios with all variables having a binary response and we also presumed there are no effect measure modifications and no other form of biases such as measurement error.

Human reproduction involves a complex selection process and the "right denominators" – time at risks of fetuses starting from conception are often missing. Using simulation technique we showed that if the exposure of interests reduces conceptions and influences fetal survival, specially affecting fetuses at high risk for the outcome, would yield a negative bias when only life born children are followed-up and examined. The magnitude of selection bias due to fetal death we estimated would generally be small, unless the exposure is a strong determinant of fetal loss and there are one or more strong risk factors of the outcome that also reduce the chance of fetal survival. For the bias mechanism we proposed, one way to reduce or even eliminate bias due to conditioning on fetal survival is to adjust for all common causes of the outcome and fetal loss in analyses which is probably not possible. But this also highlights the needs for improving our understanding

of the determinants of pregnancy loss and the importance of collecting any such data in birth cohort studies.

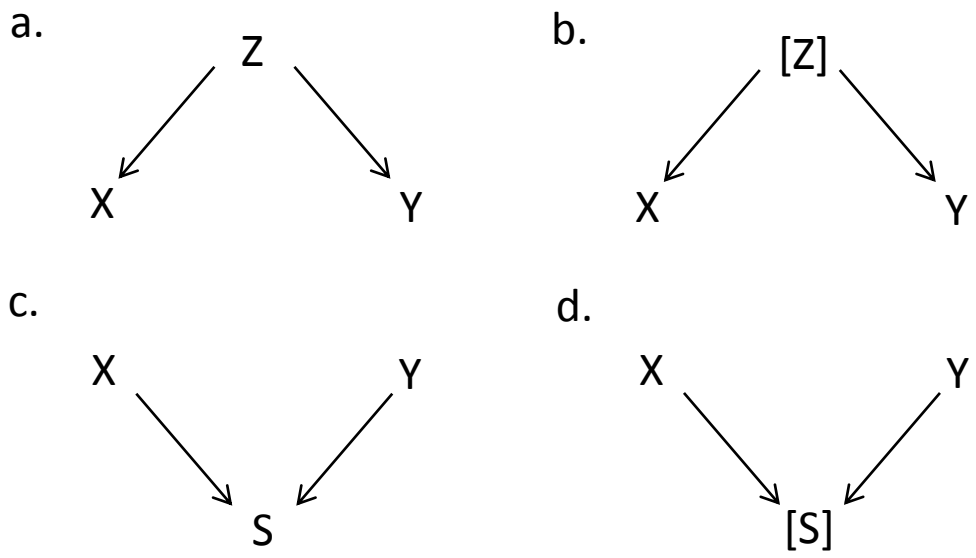
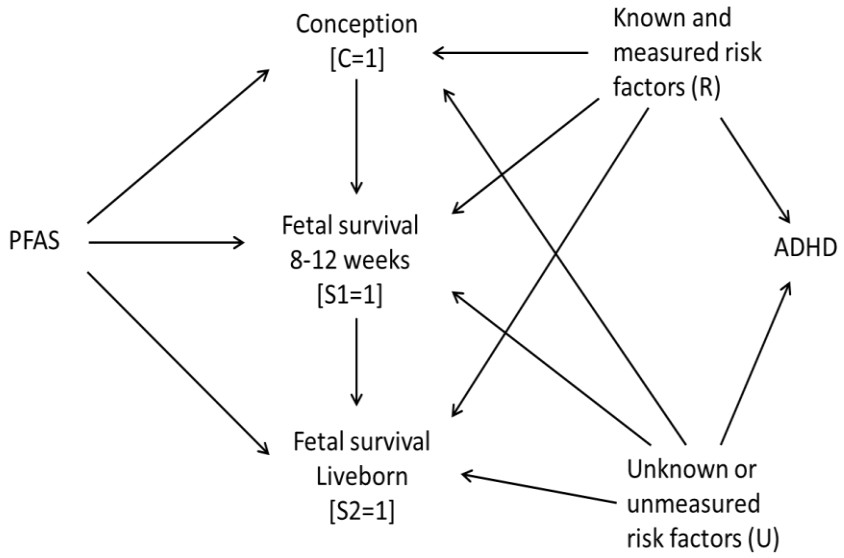


Figure 6.1 Use of the directed acyclic graphs (DAGs) to present confounding and collider bias. a. X and Y are not causally related but there is an open backdoor confounding path through Z. b. X and Y are not causally related and the backdoor path through Z is block; conditioning on Z removes the confounding. c. X and Y are not causally related, and X and Y are common cause of S. S is a collider and the path from X to Y through S is closed. d. X and Y are not causally related and X and Y are common causes of S. Conditioning on S, which is a collider, open the biasing path from X to Y through S.

a. Scenario1



b. Scenario2

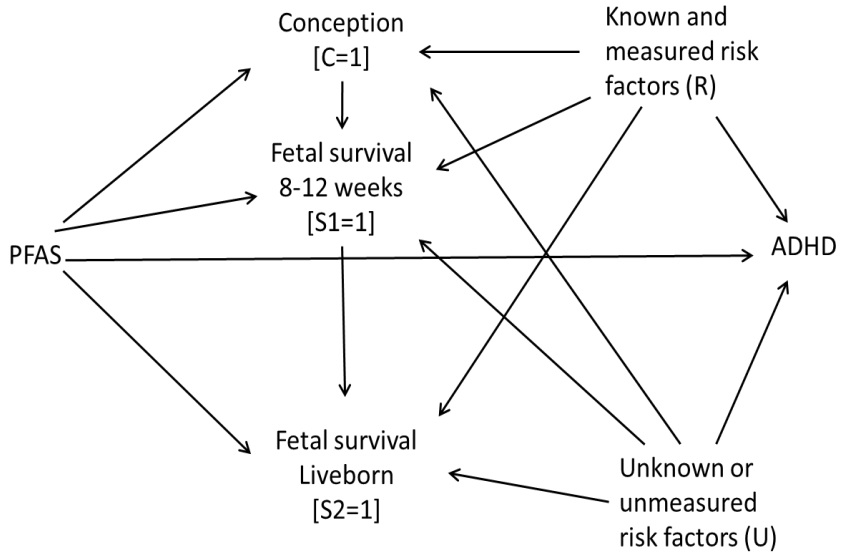


Figure 6.2 a. DAG of senario1 where no causal relationship between PFAS and ADHD in a simulated pregnancy cohort. However, conditioning on conception and fetal survival (C, S1 and S2) would open up biasing paths from PFAS to ADHD through risk factors of ADHD (R and U) b. DAG of senario2 where a causal relationship (direct effect) between PFAS and ADHD was assumed. Conditioning on C, S1 and S2 would induce similar collider biases descript in a.

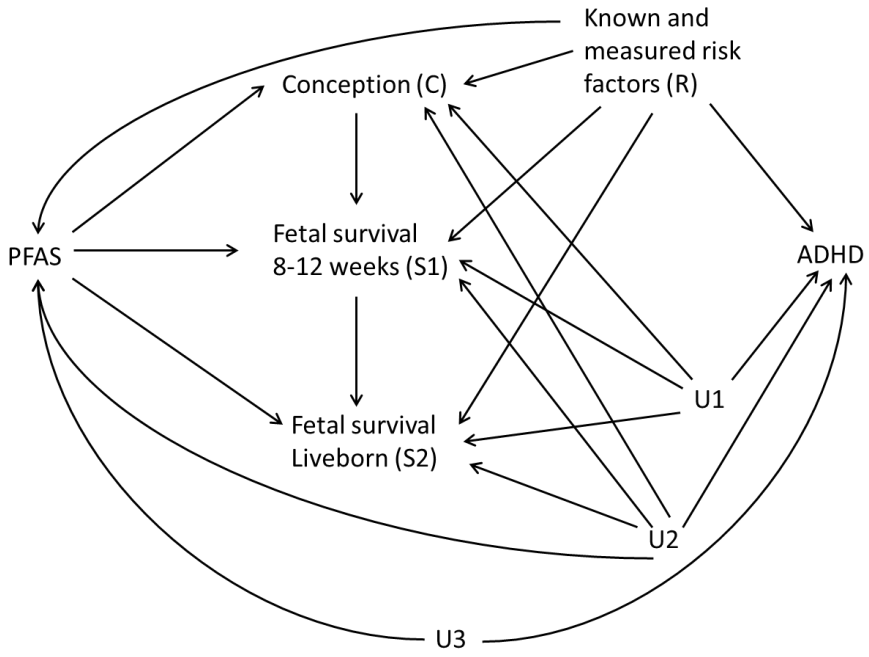


Figure 6.3 DAG of senario3 where no causal relationship between PFAS and ADHD in a simulated pregnancy cohort. Conditioning on conception and fetal survival (C, S1 and S2) would open up biasing paths from PFAS to ADHD through risk factors of ADHD (R, U1 and U2). U2 and U3 are determinants of PFAS thus would additional contribute confounding biases in this scenario.

Table 6.1 Priors for the simulation studies

Variables [a]	Abbreviation	Prevalence	specified relationships (ORs) in scenarios 1 and 2	specified relationships (ORs) in scenario 3
Perfluoroalkyl substances	PFAS	25% exposed	no causal determinants	$OR_{RF\text{-}PFAS} = 1.5$; $OR_{U2\text{-}PFAS} = 1, 1.2, 1.5$; $OR_{U3\text{-}PFAS} = 1, 2, 3, 0.5, 0.3$
Conception	C	n/a	$OR_{RF\text{-}C} = 0.5$; $OR_{PFAS\text{-}C} = 0.8, 0.5$; $OR_{U\text{-}C} = 0.8, 0.5, 0.3$	$OR_{RF\text{-}C} = 0.5$; $OR_{PFAS\text{-}C} = 0.8, 0.5$; $OR_{U1\text{-}C} = 0.3$; $OR_{U2\text{-}C} = 0.5$
Fetal survival in early gestation	S1	80% survived among all conceptions	$OR_{RF\text{-}S1} = 0.5$; $OR_{PFAS\text{-}S1} = 0.8, 0.5$; $OR_{U\text{-}S1} = 0.8, 0.5, 0.3$	$OR_{RF\text{-}S1} = 0.5$; $OR_{PFAS\text{-}S1} = 0.8, 0.5$; $OR_{U1\text{-}S1} = 0.3$; $OR_{U2\text{-}C} = 0.5$
Fetal survival at birth [b]	S2	95% survived among those survived in early gestation	$OR_{RF\text{-}S2} = 0.5$; $OR_{PFAS\text{-}S2} = 0.8, 0.5$; $OR_{U\text{-}S2} = 0.8, 0.5, 0.3$	$OR_{RF\text{-}S2} = 0.5$; $OR_{PFAS\text{-}S2} = 0.8, 0.5$; $OR_{U1\text{-}S2} = 0.3$; $OR_{U2\text{-}C} = 0.5$
Known and measured risk factors that impacted fetal survival	R	40%	no causal determinants	no causal determinants
Unknown or unmeasured risk factors	U (scenario1 and 2) ; U1,U2,U3 (scenario3)	U= 20% or 40% ; U1=10%, U2=20%, U3=20%	no causal determinants	no causal determinants
Attention-deficit/Hyperactivity disorder	ADHD	5% among live-born	$OR_{RF\text{-}ADHD} = 8$; $OR_{U\text{-}ADHD} = 2,5,10$; $OR_{PFAS\text{-}ADHD} = 2$	$OR_{RF\text{-}ADHD} = 8$; $OR_{U1\text{-}ADHD} = 5$; $OR_{U1\text{-}ADHD} = 2$; $OR_{U3\text{-}ADHD} = 1, 2$

[a] All variables were generated as binary responses (1=yes, 0=no)
[b] About 92,000 live-born were generated

Table 6.2 Simulation results of PFAS and ADHD in a hypothetical live-born birth cohort (senario1 - assuming a true null effect of PFAS on ADHD, 25% exposed to PFAS)

Pr(U=1) [c]	$OR_{U\text{-}ADHD}$	$OR_{U\text{-}C}$, $OR_{U\text{-}S1}$, $OR_{U\text{-}S2}$ = 0.8		0.5		0.3	
		Crude OR	Adjust OR[b]	Crude OR	Adjust OR[b]	Crude OR	Adjust OR[b]
where $OR_{PFAS\text{-}C}$, $OR_{PFAS\text{-}S1}$, $OR_{PFAS\text{-}S2}$ = 0.8							
0.2	2	0.98	1.00 (0.90-1.09)	0.97	0.99 (0.89-1.10)	0.97	0.99 (0.89-1.10)
0.2	5	0.97	0.99 (0.91-1.08)	0.96	0.98 (0.90-1.08)	0.95	0.97 (0.88-1.07)
0.2	10	0.97	0.99 (0.92-1.06)	0.96	0.97 (0.90-1.05)	0.94	0.95 (0.87-1.04)
0.4	2	0.97	0.99 (0.91-1.09)	0.97	0.99 (0.90-1.09)	0.96	0.98 (0.87-1.09)
0.4	5	0.97	0.99 (0.92-1.06)	0.96	0.98 (0.90-1.05)	0.94	0.96 (0.87-1.04)
0.4	10	0.97	0.99 (0.94-1.05)	0.95	0.97 (0.91-1.03)	0.93	0.94 (0.87-1.02)
where $OR_{PFAS\text{-}C}$, $OR_{PFAS\text{-}S1}$, $OR_{PFAS\text{-}S2}$ = 0.5							
0.2	2	0.92	0.99 (0.89-1.11)	0.90	0.98 (0.87-1.09)	0.90	0.97 (0.86-1.08)
0.2	5	0.91	0.98 (0.89-1.07)	0.87	0.94 (0.85-1.04)	0.85	0.91 (0.82-1.00)
0.2	10	0.90	0.97 (0.90-1.06)	0.85	0.91 (0.84-0.99)	0.81	0.86 (0.78-0.94)
0.4	2	0.92	0.99 (0.89-1.10)	0.89	0.97 (0.86-1.08)	0.87	0.94 (0.83-1.06)
0.4	5	0.91	0.97 (0.90-1.06)	0.85	0.92 (0.84-1.00)	0.81	0.86 (0.77-0.95)
0.4	10	0.90	0.97 (0.91-1.04)	0.84	0.90 (0.83-0.96)	0.77	0.81 (0.74-0.89)

[a] Assume fix priors for Pr(R=1) = 0.4, $OR_{R\text{-}C}$, $OR_{R\text{-}S1}$, $OR_{R\text{-}S2}$ = 0.5, $OR_{R\text{-}ADHD}$ = 8
[b] Adjusting for R (known and measured risk factors that impacted on fetal survival)
[c] Prevalence of unknown or unmeasured risk factors

Table 6.3 Simulation results of PFAS and ADHD in a hypothetical live-born birth cohort (senario2 - assuming a true causal OR=1.2 of PFAS on ADHD, 25% exposed to PFAS)

$Pr(U=1)$ [c]	$OR_{U\text{-}ADHD}$	$OR_{U\text{-}C}$, $OR_{U\text{-}S1}$, $OR_{U\text{-}S2}$ = 0.8		0.5		0.3	
		Crude OR	Adjust OR[b]	Crude OR	Adjust OR[b]	Crude OR	Adjust OR[b]
where $OR_{PFAS\text{-}C}$, $OR_{PFAS\text{-}S1}$, $OR_{PFAS\text{-}S2}$ = 0.8							
0.2	2	1.17	1.20 (1.08-1.31)	1.16	1.19 (1.08-1.32)	1.16	1.19 (1.08-1.30)
0.2	5	1.15	1.18 (1.09-1.28)	1.14	1.17 (1.07-1.28)	1.13	1.16 (1.05-1.26)
0.2	10	1.14	1.17 (1.09-1.25)	1.12	1.15 (1.07-1.24)	1.10	1.13 (1.04-1.23)
0.4	2	1.16	1.19 (1.10-1.30)	1.16	1.19 (1.08-1.30)	1.15	1.18 (1.06-1.30)
0.4	5	1.15	1.18 (1.11-1.26)	1.13	1.16 (1.08-1.25)	1.11	1.14 (1.04-1.23)
0.4	10	1.13	1.17 (1.11-1.23)	1.11	1.14 (1.07-1.22)	1.08	1.11 (1.03-1.19)
where $OR_{PFAS\text{-}C}$, $OR_{PFAS\text{-}S1}$, $OR_{PFAS\text{-}S2}$ = 0.5							
0.2	2	1.10	1.19 (1.07-1.32)	1.08	1.17 (1.08-1.30)	1.08	1.16 (1.04-1.29)
0.2	5	1.08	1.17 (1.07-1.27)	1.04	1.12 (1.02-1.23)	1.01	1.08 (0.98-1.19)
0.2	10	1.06	1.14 (1.06-1.23)	1.00	1.07 (0.99-1.17)	0.95	1.02 (0.94-1.11)
0.4	2	1.09	1.19 (1.08-1.31)	1.06	1.16 (1.03-1.29)	1.05	1.13 (1.01-1.26)
0.4	5	1.07	1.16 (1.08-1.26)	1.01	1.09 (1.01-1.19)	0.96	1.03 (0.93-1.13)
0.4	10	1.05	1.14 (1.07-1.22)	0.98	1.05 (0.98-1.13)	0.90	0.96 (0.88-1.03)

[a] Assume fix priors for Pr(R=1) = 0.4, $OR_{R\text{-}C}$, $OR_{R\text{-}S1}$, $OR_{R\text{-}S2}$ = 0.5, $OR_{R\text{-}ADHD}$ = 8

[b] Adjusting for R (known and measured risk factors that impacted on fetal survival)

[c] Prevalence of unknown or unmeasured risk factors

Table 6.4 Simulation results of prenatal PFAS levels and ADHD in a hypothetical liveborn birth cohort (senario2 - assuming a true causal OR=1.5 of PFAS on ADHD)

$Pr(U=1)$ [c]	$OR_{U\text{-}ADHD}$	$OR_{U\text{-}C}$, $OR_{U\text{-}S1}$, $OR_{U\text{-}S2}$ = 0.8		0.5		0.3	
		Crude OR	Adjust OR[b]	Crude OR	Adjust OR[b]	Crude OR	Adjust OR[b]
where $OR_{PFAS\text{-}C}$, $OR_{PFAS\text{-}S1}$, $OR_{PFAS\text{-}S2}$ = 0.8							
0.2	2	1.45	1.50 (1.38-1.62)	1.45	1.49 (1.36-1.62)	1.44	1.48 (1.36-1.63)
0.2	5	1.42	1.47 (1.36-1.57)	1.41	1.45 (1.34-1.56)	1.39	1.44 (1.33-1.56)
0.2	10	1.37	1.42 (1.33-1.51)	1.36	1.40 (1.31-1.50)	1.34	1.38 (1.29-1.49)
0.4	2	1.44	1.49 (1.37-1.61)	1.43	1.48 (1.37-1.61)	1.42	1.47 (1.34-1.62)
0.4	5	1.40	1.46 (1.37-1.55)	1.39	1.44 (1.34-1.54)	1.36	1.41 (1.30-1.53)
0.4	10	1.36	1.41 (1.34-1.49)	1.33	1.39 (1.30-1.46)	1.30	1.35 (1.26-1.44)
where $OR_{PFAS\text{-}C}$, $OR_{PFAS\text{-}S1}$, $OR_{PFAS\text{-}S2}$ = 0.5							
0.2	2	1.37	1.49 (1.36-1.63)	1.34	1.46 (1.33-1.61)	1.34	1.45 (1.30-1.61)
0.2	5	1.33	1.45 (1.34-1.57)	1.27	1.39 (1.27-1.51)	1.25	1.35 (1.22-1.48)
0.2	10	1.28	1.39 (1.30-1.50)	1.21	1.31 (1.21-1.42)	1.16	1.25 (1.14-1.37)
0.4	2	1.36	1.48 (1.35-1.61)	1.32	1.44 (1.31-1.59)	1.30	1.42 (1.26-1.57)
0.4	5	1.31	1.44 (1.34-1.53)	1.24	1.36 (1.25-1.47)	1.18	1.28 (1.16-1.39)
0.4	10	1.26	1.38 (1.31-1.47)	1.18	1.28 (1.20-1.37)	1.09	1.17 (1.08-1.26)

[a] Assume fix priors for $Pr(R=1) = 0.4$, $OR_{R\text{-}C}$, $OR_{R\text{-}S1}$, $OR_{R\text{-}S2} = 0.5$, $OR_{R\text{-}ADHD} = 8$

[b] Adjusting for R (known and measured risk factors that impacted on fetal survival)

[c] Prevalence of unknown or unmeasured risk factors

Table 6.5 Simulation results of prenatal PFAS levels and ADHD in a hypothetical liveborn birth cohort (senario3 - assuming no effect of PFAS on ADHD)

$OR_{U2\text{-}PFAS}$	$OR_{U2\text{-}ADHD}$	$OR_{U3\text{-}PFAS}$	$OR_{U3\text{-}ADHD}$	$OR_{PFAS\text{-}C}$, $OR_{PFAS\text{-}S1}$, $OR_{PFAS\text{-}S2,}$ = 0.8	0.5
				Adjust OR[b]	Adjust OR[b]
1	**2**	**1**	**1**	0.97 (0.88-1.07)	0.92 (0.82-1.02)
1.2	2	1	1	0.99 (0.90-1.09)	0.93 (0.83-1.04)
1.5	2	1	1	1.01 (0.91-1.11)	0.95 (0.84-1.06)
1.5	2	2	2	1.10 (1.01-1.20)	1.04 (0.94-1.15)
1.5	2	2	4	1.23 (1.14-1.32)	1.13 (1.03-1.24)
1.5	2	0.5	2	0.94 (0.85-1.03)	0.88 (0.79-0.98)
1.5	2	0.5	4	0.85 (0.78-0.93)	0.80 (0.73-0.89)

[a] Assume fix priors for Pr(R=1)=0.4, Pr(U1=1)=0.1, Pr(U2=2)=0.2, Pr(U3=2)=0.2, $OR_{R\text{-}C}$, $OR_{R\text{-}S1}$, $OR_{R\text{-}S2}$ = 0.5, $OR_{R\text{-}ADHD}$ = 8, $OR_{U1\text{-}C}$, $OR_{U1\text{-}S1}$, $OR_{U1\text{-}S2}$ = 0.3, $OR_{U2\text{-}C}$, $OR_{U2\text{-}S1}$, $OR_{U2\text{-}S2}$ = 0.5, $OR_{U1\text{-}ADHD}$ = 8, $OR_{U2\text{-}ADHD}$ = 2

[b] Adjusting for R (known and measured risk factors that impacted on fetal survival)

Chapter VII: Overall Summary and Discussion

7.1 Strengths and limitation

In our study, PFAS values were measured in prospectively collected maternal plasma samples. The laboratory at Aarhus University, Department of Environmental Science has been certified by INSPQ Centre de toxicology (Quebec) in using the most advanced techniques to date in PFAS measurements. Moreover, PFAS measurements are obtained under strict quality controls procedures. High correlations between PFAS values was found when comparing PFAS concentrations of the same samples measured in different laboratories. Previous studies also have shown that PFASs in maternal plasma correlated with cord blood PFASs and suggested that it is a marker of fetal exposure, although cord blood is drawn later than the outcome's etiological window during gestation.

Another major strength of this study is the availability of nationwide comprehensive hospital registries for outcomes assessment. In our source population with as much as 13 years of follow up, only less than 2% of children were considered lost to follow-up due to death or emigration strongly reducing the chance of selection bias due to passive follow-up that did not require subject response. Moreover, outcomes of Autism and ADHD were identified from both the Danish National Hospital Registry (NPR) as well as from the Danish Psychiatric Central Registry (PCR). Previous study in Denmark that only used NPR without linkage from PCR may have underestimated the diagnoses of ADHD in this study population (Greene et al. 2011). Diagnoses of childhood autism in the psychiatric registry have previously been shown to have high validity (Lauritsen et al. 2010), but reliability of the diagnosis of ADHD in the NPR and PCR are unknown. Children with ADHD treated in hospitals are likely more severe cases and may also be affected by

multiple morbidities, while less severe ADHD cases could possibly to be only treated by child psychiatrists in the community; these children thus might not be included in the hospital registry system (Li et al. 2010).

The outcome CP is determined from the Danish National Cerebral Palsy Register (DNCPR), which thoroughly evaluates doctors' diagnoses from hospital registries. Incomplete registration of CP is not very likely given that treatment in Danish hospitals is free of charge and the severity of the condition of cerebral palsy. However, the inclusion in the DNCPR is based on information about the children at the age of 4-6 years. Children have to survive to at least one year of age to be diagnosed in the DNCPR, therefore we likely have under-ascertained severe CP cases who died in pregnancy, at birth, or during infancy. As we have shown in the bias analysis in Chapter VI, if exposure to PFAS reduces fetal/neonatal survival, selection bias may occur and we would expect an attenuation of the observed results.

The PFAS levels in the DNBC are comparable to the U.S. general population during the study period, but the concentrations may not be representative for the exposure patterns typical in other countries. For instance, in Taiwan long-chain PFASs including perfluoroundecanoic acid (PFUnDA) and perfluorododecanoic acid (PFDoDA) were found to be in high concentrations in pregnant women during the similar period of our study (Wang et al. 2014). Concentrations of long-chain PFASs were also suggested to exceed the level of 8-carbon PFOA in the Japanese, Korean and Vietnamese general population during 2000-2008 (Harada et al. 2011). These PFASs were rarely detected in our study population and thus not studied. Moreover, our results also may not be generalized to workers or communities exposed to high level of PFASs (Frisbee et al.

2010, Olsen et al. 2003). PFASs exposure patterns have changed in the last ten years both in Europe and in the USA, especially following the drop in PFOS and PFOA production since 2000 and the increasing use of substitute PFASs. We were unable to study these emerging substitute PFASs using our data.

Due to the high costs of measuring PFAS and limited samples availability, we were only able to investigate a subset of the DNBC based on a case-cohort design, limiting the statistical power of our effect estimates. Confidence intervals were broad in some sub-group analyses such as the assessment of potential effect measure modification by child's sex. Moreover, we have no data for other endocrine disrupting chemicals and therefore we could not evaluate possible "mixture effect" with other co-occurring endocrine disruptors.

Approximately 30% of all pregnant women in Denmark during the recruitment period were included in the cohort; approximately half of those who did not participate were never invited because their general practitioners did not take part in the study. Non-participation in the DNBC cohort has been shown to probably have small if any effects on internal validity, but it may limit the generalizability of the results (Nohr et al. 2006). Since participants in the DNBC are ethnically homogeneous our results may not be generalizable to ethnicities other than North-Europeans, especially if race and genetic factors modify the associations between PFASs and neurological diseases in children.

7.2 Public Health Relevance

This study is part of the FETOTOX program (http://fetotox.au.dk/fetotox/) that consists of several studies aiming to estimate effects for a wide range of Persistent Organic Pollutants exposures, with a focus on PFASs, and lifestyle factors on health of newborns and children. Our results are important parts for the FETOTOX program to meet the objectives of:

a) providing new knowledge on susceptible periods of fetal development, and understand interrelationship between the mother-child environment and lifestyle, and perhaps prevent neurological and neurodevelopmental diseases
b) contribute to early warning systems on fetal toxicity
c) compare the Danish study with the international birth cohorts in Norway, Greenland and China on POP/PFAS levels in maternal blood and birth outcomes.
d) serve as documentation for policy making addressing environmental health risks.

Serious actions have been taken by the U.S. Environmental Protection Agency (EPA) to regulate PFASs. EPA enacted the on-going Long-Chain Perfluorinated Chemicals Action Plan (EPA 2009) with the goal to examine and review the potential risks of human exposure due to PFASs and formulate regulation policy. For instance on September 30, 2013, EPA issued a rule requiring companies to report all new uses of long-chain perfluoroalkyl carboxylates as part of carpets. The action plan also emphasized the importance of evaluating the potential of PFASs disproportionate impact on the developing fetus and children. Following the voluntary phase-out of PFOS in production by 3M company, EPA had an agreement with eight fluoropolymer and -telomere manufacturers on a PFOA-stewardship program with the goal to eliminate PFOA

production by 2015 (EPA 2012a). In addition, EPA is also committed to continue evaluating the alternatives of PFASs under New Chemicals Program (EPA 2012b) and collaborate with other countries in managing PFASs globally.

In May 2009 PFOS was included in Annex B of the Stockholm Convention as a persistent organic pollutants (Convention 2011). Canada prepared a risk management scope for PFOA and long chain PFASs in 2010 (Canada 2010). A Europe-wide regulation is currently missing, but Norway had proposed to ban PFOA in consumer products (Commission 2010), and in Germany maximum concentrations for drinking water were recommended (Agency 2011). We hope our results will contribute additional scientific evidence for policy makers who responsible for PFASs regulations worldwide.

Due to regulatory activities during the last years in the US, Canada and Europe, industry has been shifting the use of long-chain PFASs to shorter chain PFASs. Although the short-chain PFASs are expected to be less persistent, the short-chain PFASs have higher mobility to be transferring into groundwater. It was suggested that the short-chain PFASs contribute >50% of the total PFASs in groundwater (Eschauzier et al. 2013). To date no efficient technologies are known to remove the short-chain PFASs from water, and increasing human exposure to short-chain PFASs exposure via drinking water may become an issue in future. It is thus very important to continue monitoring human exposures to both long- and short-chain PFASs, and conduct evaluations of their potential long-term health impacts.

We identified a positive association between prenatal exposures to PFASs and risk for CP in boys, and this finding may have important public health implications. First, we showed

that exposure to environmental chemicals or endocrine disruptors during pregnancy may be relevant to the etiology of CP which is novel. Future studies should investigate whether other chemicals such as pesticides, phthalates and bisphenol A (BPA) are also associated to CP risk. Experimental studies should be conducted to reveal possible mechanistic pathways of this association. If the link between PFASs and CP is causal, strategies should be developed to protect pregnant women and fetuses from PFAS exposures to prevent CP. Prevention of a permanent neurological and movement disorder like CP that has long-lasting patient, care-giver, and societal impacts is very important. On the other hand, we did not find a consistent pattern of maternal PFASs and ADHD or autism risk in children, and the search for avoidable environmental causes of ADHD or autism should continue. It is important prevent or slow down the increasing incidence of both diseases.

7.2 Perspectives for future research

PFASs and neuropsychological measures in children at 5 years of age

About 1800 children at the age of 5 and their mothers from the DNBC participated in the "Lifestyle During Pregnancy Study (LDPS)" (Kesmodel et al. 2010). These children were examined with extensive psychological testing of different brain functions, including neuropsychological test battery and questionnaires filled out by both the parents and staff at child care centers (tests included WPPSI, TEACH-%, Sternberg Task, BRIEF, SDQ, draw a person and physical examinations, motor development and audiometry). We can use these data and evaluate the association between prenatal exposure to PFASs and children's cognitive function.

PFAS and fetal losses

Animal studies suggested that prenatal exposure to higher levels of PFASs associated with fetal and neonatal deaths. With additional sampling and PFASs measurement, we can evaluate the relationship between PFASs and spontaneous abortion in the DNBC, which has not been examined in human studies to date. Moreover, PFASs' impact on conception and early fetal losses in human are still under debate (Kristensen et al. 2013, Fei 2009, Whitworth et al. 2012, Vestergaard et al. 2012, Buck Louis et al. 2013). A previous study using a subset from DNBC reported a strong association between higher PFASs and longer waiting time to pregnancy in women (Fei 2009). A longer time to pregnancy may indicate lower fecundability, and suggest that exposure to PFASs reduces the chances to conceive or increases the rate of early fetal loss. However, since PFASs are able to transfer from mothers to fetuses, a recent study raised the possibility that the observed association may be explained by reverse causality such that parous women who have a long inter-pregnancy interval might have longer time to accumulate PFASs in their body (Whitworth et al. 2012). We can combine the FETOTOX population controls and the earlier DNBC samples, and re-evaluate the association between PFASs and waiting time to pregnancy among nulliparous women only and stratify on maternal age which is a strong predictor of PFASs levels and fecundability of the mothers.

PFASs, maternal weight gain during pregnancy, and childhood obesity

PFASs were repeatedly shown to interact with lipids and cholesterols in animals and in human (Fletcher et al. 2013). These finding raised the possibility that PFASs might increases weight gain both in the mothers and in the children. A DNBC study found no association between prenatal PFASs level and children BMI at 7 years of age, but this

association should be re-examine using children BMI data at 11 years of age since children weight changes might be influenced by PFASs in puberty but not before (data collection is expected to be ready at the end of 2014). No study to date has examined the relationship of PFASs and maternal weight gain during pregnancy.

7.3 Concluding remarks

In summary, we found that prenatal exposure to PFASs increases the risks of CP in boys. Exposure to environmental chemicals or endocrine disruptors including PFASs during pregnancy may be relevant to the etiology of CP. More research is needed to understand the potential causal mechanisms between PFASs and CP. We did not find consistent associations between maternal PFAS levels and ADHD or autism in children, but future studies that search for other endocrine disruptors that may contribute to disease etiology of ADHD and autism are recommended. In bias analysis, we quantitatively showed that conditioning on life-born status in observational study can yield a negative bias if an exposure of interests reduces conceptions or fetal survival. Our findings are important and may serve as information for regulation related policies that address potential health risks due to PFASs.

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