Exposure, Placental Transfer, and Neurodevelopmental Effects of Bisphenol A (BPA) and BPA-Alternatives

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Abstract

Bisphenol A (BPA) is an endocrine disruptor widely used in plastics and thermal receipt paper, and frequently detectable in human biofluids. Human studies have shown associations between maternal BPA exposure and developmental effects in children. BPA-alternatives, including bisphenol S (BPS) and bisphenol F (BPF) have now been widely used, yet study on these alternatives remains limited.

In order to evaluate the *in utero* exposure of BPA metabolites and alternatives, the major BPA metabolites and BPA-alternatives were quantified in 61 pairs of maternal and cord sera from Chinese participants. Total BPS was only detectable in 4 maternal and 7 cord sera, indicating low exposure for this population. Total BPA metabolites in cord serum were significantly higher than in maternal serum. Unlike in oral exposure studies where BPA-glucuronide is the major BPA metabolite, here BPA-sulfate was the dominant metabolite, in both maternal and cord sera.

To evaluate the pharmacokinetics of dermal BPA absorption, six male participants handled receipts containing isotope-labeled BPA-d_{16} for 5 min, followed by hand-washing 2 hrs later. Urine (0-48 hrs) and serum (0-7.5 hrs) were monitored for free and total BPA-d_{16}. One week later, participants took a dietary administration with monitoring as above. One participant repeated the dermal administration with extended sample monitoring. After dietary exposure, urine total BPA-d_{16} peaked within 5 hrs and quickly cleared within 24 hrs. After dermal exposure, urine concentrations only peaked after 15-34 hrs, cumulative excretion increased linearly for 2 days, and urinary total BPA-d_{16} was detectable after 1 week. In follow up dermal exposure, the participant showed linear cumulative excretion over 5 days, detected urinary total BPA-d_{16} after 9 days, and detected serum free BPA-d_{16} after 1 and 2 days. The proportions of urinary free BPA-d_{16} following dermal exposure (range: 1.6 - 2.9% of total BPA-d_{16}) were much higher than in the dietary exposure (range: 0.4 - 1.0%).

To examine the exposure and sources of BPA and BPA alternatives among pregnant women, free and total BPA, BPS and BPF were measured in 467 second trimester maternal urine samples, and in 455 paired samples collected 3 months postpartum. Free BPA, BPS and BPF were detectable in <2% of second trimester maternal samples. Nevertheless, maximum concentrations (Cmax) of free BPS and BPF were detected in samples with the highest total BPS and BPF respectively, and the proportion of free bisphenols was always <1%. Geometric mean total BPA in second trimester urine and postpartum urine was 5-7 times higher than total BPS. However, Cmax of total BPA pre- and post-partum (44 ng/mL, 55 ng/mL) was much lower than Cmax for total BPS (240 ng/mL, 72 ng/mL) or BPF (390 ng/mL, 120 ng/mL) The Cmax of estimated 24hr intake of BPS (14 nmol/kg BW/d) and BPF in 1% of women (21-30 nmol/kg BW/d) approached and even exceeded the tolerable daily intake of BPA (18 nmol/kg BW/d). Consumption of canned food, particularly canned meat, was associated with higher urinary total BPA, but not associated with urinary BPS.

To examine the effects of maternal BPA and BPS exposure on subsequent child neurodevelopment at two years of age, neurodevelopment was assessed by the Bayley-III scales (n=394), and behavioural syndromes were evaluated using the Child Behaviour Checklist (CBCL) (n=358). Potential confounders include neurotoxic metals and maternal nutrient status/intake in pregnancy. After adjusting for covariates, increasing BPA exposure was associated with poorer Social Emotional in boys and more behavioural problems in all children, with aggressive behaviour and externalizing problems mainly observed in girls. Higher maternal status of methyl donors (folate, vitamin B12 and choline) lessened the adverse effects of BPA exposure on CBCL scores. Higher BPS exposure was associated with lower Motor scores for girls only, more sleep problems in all children and more aggressive behaviours in girls.

Based on the results in this dissertation, I concluded that: the human fetus and pregnant mother have unique exposure to BPA metabolites; a single dermal contact could lead to prolonged BPA exposure and less detoxification compared to the oral pathway; canned food is a dominant exposure

source for BPA, and dermal exposure should be evaluated for BPS; both BPA and BPS have sex-specific effects on child development and behaviour, while higher intake of methyl donors in pregnancy counteracted some adverse effects of BPA.

Preface

The research discribed in Chapter 2 was an international research collaboration with Dr. Yoning Wu, Dr. Yunfeng Zhao and Dr. Jingguang Li at the China National Center for Food Safety Risk Assessment. Parts of Chapter 2 have been published as Jiaying Liu, Jingguang Li, Yongning Wu, Yunfeng Zhao, Fengji Luo, Shuming Li, Lin Yang, Elham K. Moez, Irina Dinu, Jonathan W. Martin, "Bisphenol A metabolites and bisphenol S in paired maternal and cord serum," *Environ. Sci. Technol.* **2017**, 51(4), 2456-2463. I was responsible for the experimant design, sample analysis, data analysis, and manuscript composition. Dr. Jonathan W. Martin was the supervisory author and was involved with study design and manuscript writing. Dr. Yoning Wu, Dr. Yunfeng Zhao and Dr. Jingguang Li were responsible for the sample collection and provided the lab resources for analyzing these serum samples in China. Dr. Fengji Luo, Dr. Shuming Li in Chaoyang District Center for Disease Control and Prevention provided the lab resources for the sample preparation. Dr. Lin Yang in China National Center for Food Safety Risk Assessment contributed to the blank test of sampling materials. Dr. Irina Dinu and Dr. Elham K. Moez at University of Alberta assisted with the data analysis. The protocol of this study was reviewed and approved by the ethics committee of the China National Center for Food Safety Risk Assessment (approved on Feb. 15, 2013) and by the Health Research Ethics Board of the University of Alberta (ID: Pro00038394).

The study protocol of Chapter 3 was reviewed and approved by the Health Research Ethics Board of the University of Alberta (ID: Pro00051789). Parts of this Chapter have been published as Jiaying Liu, Jonathan W. Martin, "Prolonged exposure to bisphenol A from single dermal contact events," *Environ. Sci. Technol.* **2017**, 51(17), 9940-9949. I was responsible for study design, volunteer recruitment, data collection, data analysis and manuscript composition. Dr. Jonathan W. Martin was involved with study design, participated in pilot-testing, and contributed to manuscript writing and revisions. Dr. Irina Dinu and Elham K. Moez (School of Public Health, University of Alberta) provided assistance in statistical analysis of the data.

The studies in the APrON cohort, described in Chapters 4 and 5 was a research collaboration with Dr. Deborah Dewey in University of Calgary, Dr. Catherine Field and Dr. Irina Dinu in University of Alberta, and the APrON team played important roles in establishing the cohort and subsequent data collection and integration. The participant recruitment and data collection were led by Dr. Deborah Dewey and Dr. Catherine Field. Dr. Irina Dinu and Dr. Leah J. Martin (Public Health Agency of Sweden) assisted with the data analysis. I was responsible for the trace biofluid analysis and for conducting the data analysis. The

study protocol for APrON was approved by the Health Research Ethics Board of the Universities of Alberta (Study ID: Pro00002954) and Calgary (Ethics ID: REB14-1702_REN3).

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BPA	bisphenol A; 4,4'-propane-2,2-diyldiphenol
BPA-d_{16}	deuterated bisphenol A
$^{13}C_{12}$-BPA	Bisphenol-A-(diphenyl-$^{13}C_{12}$)
BPS	Bisphenol S; bis(4-hydroxyphenyl) sulfone
BPF	4,4'-dihydroxydiphenylmethane
BPAF	4,4'-(hexafluoroisopropylidene) diphenol
BPB	2,2-bis(4-hydroxyphenyl)butane
BPs	bisphenols
BPA-glucuronide	bisphenol A mono-beta-D-glucuronide
BPA-sulfate	bisphenol A sulfate (dodium salt)
BPA-disulfate	bisphenol A bissulfate (disodium salt)
$^{13}C_{12}$-BPA-glucuronide	isotopically labeled bisphenol A mono-β-D-glucuronide
GM	geometric mean
LOD	limits of detection
LOQ	limit of quantification
RSD	relative standard deviation
CI	confidence interval
SD	standard deviation
Cmax	maximum concentrations
Tmax	sampling time of maximum concentrations
UC_{crea}	urinary creatinine adjusted concentration
$CE_{smoothed}$	24-hr urinary creatinine excretion
BW	body weight
P	participant
hrs	hours
d	day
SPE	solid phase extraction
HPLC	High performance liquid chromatography

NH_4OH	ammonium hydroxide
TDI	tolerable daily intake
EDI	estimated daily intake
MADL	maximum allowable dose level
APrON	Alberta Pregnancy Outcomes and Nutrition
EPA	US Environmental Protection Agency
EFSA	European Food Safety Authority
NHANES	National Health and Nutrition Examination Survey
OEHHA	Office of Environmental Health Hazard Assessment
ER	Estrogen receptors
K_{ow}	Octanol-water partition coefficient
K_{oc}	Soil organic carbon-water partition coefficient
AR	Androgen receptor
TR	Thyroid hormone receptor
T_3	Thyroid hormone
Bayley III	Bayley Scales of Infant Development – Third Edition
CBCL	Child Behaviour Checklist
BMI	birth mass index
Cu	Copper
Zn	zinc
Mn	manganese
Se	selenium
DHA	docosahexaenoic acid
AA	arachidonic acid
As	arsenic
Cd	cadmium
Pb	lead
Hg	total mercury

1 Introduction

1.1 BPA uses:

Bisphenol A (BPA) is a high production volume chemical used for polycarbonate plastic manufacturing, epoxy resins, and as a developer in thermal paper, such as for shopping receipts and luggage tags at airports. [1] BPA-based polycarbonate is used in food and liquid containers such as microwave ovenware, beverage bottles, reservoirs for water dispensers, water pipes, and in other non-food applications such as toys, pacifiers and medical devices such as eyeglass lenses, intravenous administration sets, syringes and catheters. [2,3] BPA-based epoxy resins are used as linings of food and beverage cans, as a coating on residential drinking water storage tanks, and in resin-based powder paints, printing inks, flame retardants and for medical purposes including dental composite and sealant materials.[2,4,5]

In 2003, global production of BPA was 3.2 million metric tons.[6] By 2013, the global demand for BPA was predicted to be 7.0 million metric tons and is expected to reach 9.6 million metric tons by 2020 [7]. Asia accounted for 56% of the total world consumption of BPA in 2015 and the growth in demand and capacity in this region is driven by China's market.[8] North America and Western Europe, combined, accounted for 36% of global consumption of BPA in 2015.[8]

1.2 Properties of BPA:

BPA is a solid under ambient conditions and its melting point is 156-158 °C. [9] It is a weak phenolic acid with predicted acid dissociation constant (pKa) of 10.29±0.10 at 25 °C, [10] meaning it will be present as a neutral substance in most natural waters. The water solubility of BPA is 120 mg/L at 20 °C.[11] The experimentally derived log octanol-water partition coefficient (K_{ow}) and the predicted log soil organic carbon-water partition coefficient (K_{oc}) at 25 °C (pH1-7) are 3.41 and 3.36, respectively. [10,11] The vapour pressure at 25 °C is 5.21×10^{-2} mPa. [12] Thus, BPA is a low volatility compound with moderate solubility and moderate affinity for soil organic matter, and is therefore unlikely to be mobile or bioavailable in soils.[13,14] BPA's emission into the environment is generally through dissolution in water, or in the form of particulates.[9]

1.3 BPA in the environment

Due to its wide usage, BPA now occurs ubiquitously in the environment.[15] It is regarded as a pseudo-

persistent chemical, meaning that despite its short half-life it is ubiquitous due to continuous environmental emissions.[16] BPA dissolved in surface water can be rapidly degraded through both microbial biodegradation and photodegradation with a low potential of bioaccumulation in animals.[5]

1.3.1 BPA in aquatic environments

BPA has been detected in various aqueous media, including fresh and marine surface waters, ground water and wastewater treatment plant influents and effluents.[15] Observed freshwater concentrations of BPA range from <0.17 ng/L to 21 μg/L in US and China.[17–20] Flint et al. reviewed environmental BPA concentrations in different countries and concluded that observed BPA concentrations in oceans and estuaries are typically lower relative to freshwater systems.[15]

Measurements of the concentration of BPA in the sediments and suspended solids has ranged from 0.19 to 56 μg/kg dry weight.[15,19] Flint et al. reviewed studies of BPA in both water and sediments, and found that BPA concentrations were usually higher in the sediments than in the upper water column.[15]

1.3.2 BPA in ambient air and dust

Fu et al. analyzed BPA in more than 260 atmospheric aerosol samples collected from various cities in Asian and New Zealand, as well as remote sites including the Polar Regions.[21] The results showed that the concentrations of BPA (0.001 – 17 ng/m^3) in the atmosphere ranged over 4 orders of magnitude with a declining trend from the continent (except for the Antarctica) to remote sites.[21] BPA was detected in 87% of 83 indoor air samples collected in Albany, New York, USA.[22] The average and maximum BPA concentration in these bulk air samples were 0.79 and 4.7 ng/m^3, respectively.[22] Compared to these indoor and outdoor air samples, concentration of BPA was up to 6 orders of magnitude higher in air samples collected in companies that made BPA or BPA-based products[n=146; geometric mean (GM): 4.0 μg/m^3; maximum 920 μg/m^3].[23]

BPA (GM: 1.3 μg/g) was detected in the indoor dust samples (n=156) collected from US and three Asian countries, and the highest concentration was 39 μg/g detected in a indoor dust sample from Korea.[24] Wang et al. monitored BPA in 284 house dust samples from 12 countries and found that BPA was detected in 83% of the samples (>0.5 ng/g).[25] The mean and maximum concentration of BPA in these house dust samples were 1.0 μg/g and 110 μg/g, respectively.[25]

1.4 Exposure sources in humans:

Human populations are widely exposed to low levels of BPA.[26,27] According to national surveys in Canada (2012-2013) and in the United States (US) (2003-2012), approximately 90% of the general population have detectable urinary concentration of BPA (i.e. >0.2 or >0.4 ng total BPA/mL).[28,29] The exposure sources of BPA in humans may be grouped to dietary and non-dietary exposure sources.

1.4.1 Dietary exposure

Diet has long been regarded as the major human exposure pathway to BPA.[30–32] The European Food Safety Authority (EFSA) systematically reviewed the scientific literature covering the period from 2006 to December 2012.[2] These data showed differences in BPA concentrations between canned and non-canned food in a large majority of food categories, with higher BPA concentrations in the canned food. Canned food categories including "Grain and grain-based products", "Legumes, nuts and oilseeds", "Meat and meat products", "Fish and other seafood", "Herbs, spices and condiments", "Composite food", and "Snacks, desserts, and other foods" presented an average BPA concentration above 30 µg/kg. Lower levels of BPA were found in other categories, and the average BPA concentration was lower than 3 µg/kg in canned beverages (i.e. water and beverages). Among 19 non-canned food categories, the highest average BPA concentrations, 9.4 and 7.4 µg/kg, were found in "Meat and meat products" and "Fish and other seafood", respectively. Overall, among the population older than 6 months, infants and toddlers (6 months to 3 years) had the highest estimated average (375 ng/kg BW/day) and high (857 ng/kg BW/day) dietary exposures.

High concentrations of BPA were also found in canned food in Canada, whereas BPA was low or below limits of detection (LOD) in most non-canned food in the Canadian total diet study (2008-2012).[33] The highest concentration of BPA was found in canned soup containing meat, with a concentration in the range of 21 to 104 ng/g.[33] The average daily intake of BPA from food was estimated to be 0.055 µg/kg BW/day for the general population in Canada.[34]

1.4.2 Non-dietary exposure

There is evidence that non-dietary exposures to BPA may be underestimated. For example, the biological half-life of BPA following oral exposure is less than 6 hrs [35,36] but Stahlhut et al. found that urinary BPA concentrations did not decline rapidly with fasting-times in 1,469 participants from the 2003-2004 National Health and Nutrition Examination Survey (NHANES).[37] These findings in the U.S. suggested substantial non-dietary BPA exposure pathways in the general population.[37]

Due to the high levels of BPA detected in paper products, including thermal paper receipts and paper currencies,[38–43] dermal exposure to BPA may be an important non-dietary exposure pathway in some people. Data from NHANES supports the link between occupational exposure from thermal paper and increased urinary BPA excretion.[44] Higher urinary BPA levels in cashiers have also been observed previously,[45,46] and recently the dermal contact route was estimated to contribute 52 - 84% of total BPA exposure for cashiers.[47] For the general population, the latest exposure assessment from the EFSA concluded that thermal paper was the second most important source of BPA in all population groups above 3 years of age, and quantitatively was thought to contribute 7-15% of total average exposure.[2] It should be noted that the absorption fraction of 30% was used for dermal exposure estimates from thermal paper, which might underestimate the actual dermal absorption.[2]

Besides dermal exposure, other non-dietary exposure sources might also include inhalation of indoor air, ingestion of dust, dental materials, toys intended to be mouthed, and cosmetics. Resin-based dental sealants and composites are possible exposure sources of BPA,[48] however, no significant association was found between urinary BPA concentration and number of dental sealants.[49,50] As summarized in the section of 1.3.2, BPA was also frequently detectable in indoor air and dust,[25,51] although the contribution of dust to daily intakes of BPA was estimated to be a minor (<10%) proportion of estimated daily intake (EDI) in countries such as China and USA.[25] BPA concentrations in outdoor air vary widely and depend on regional factors.[21] Thus, it is difficult to accurately estimate the extent of exposure to BPA from air and dust.[2]

1.5 Toxicology of BPA

1.5.1 Vitro studies:

The toxicological hazards of BPA include its activity as an endocrine disruptor through pathways involving the estrogen and androgen receptors, the thyroid hormone receptor, and peroxisome proliferator-activated receptor-γ.[27,52–57] BPA competes with estradiol for binding to estrogen receptors (ER) in rat uterus,[58] although the affinity between BPA and ER is considered weak, 15,000-fold less than estradiol.[59] By using a yeast-based assay, Lee et al. found that BPA had antiandrogenic activity at multiple steps of androgen receptor (AR) activation and function.[52] The antiandrogenic activity of BPA was also observed in an AR-mediated reporter gene assay system.[53] For thyroid hormone receptor (TR) prepared from the Sprague Dawley rat liver, BPA acted as a competitive antagonist by displacing thyroid hormone (T_3) and activating gene transcription that is normally suppressed by T_3.[55] BPA has also been

shown to stimulate the peroxicome proliferator-activated receptor-γ in human ovarian granulosa cells, which suggests a potential role of BPA in steroidogenesis and proliferation within the ovarian follicular compartment.[57]

1.5.2 Animal studies:

Based on the hypothesis that BPA may, as do many hormones and xenobiotics, display a U-shaped dose-response curve,[60–64] recent studies in animal models have evaluated possible biological effects at low and environmentally relevant concentrations. Thus, in following sections, I mainly reviewed the animal studies using environmentally relevant administration dosage. As dietary exposure is regarded as the major pathway of human BPA exposure, oral administration is mainly used in these animal studies and adverse effects observed following subcutaneous dosage have been discounted. However, considering the low metabolism efficiency following dermal exposure (Chapter 3), subcutaneous BPA exposure in animal studies may not necessarily be an unrealistic model for consideration in risk assessments.

1.5.2.1 Effects on metabolism and obesity:

After low-dose oral administration of BPA at a dose of 5 μg/kg BW/day to pregnant Wistar rats during gestation and lactation, and to F1 offspring lifelong, changes in sweet preference and salt and fat solution intakes, and increase in body weight were observed in the F2 generation.[65] Low-dose BPA oral exposure (at a dose grossly corresponding to the tolerable daily intake (TDI) of BPA) induced sex-dependent hepatic metabolic disorders in the progeny of obese mice without toxicity and weight gain.[66] Low-dose subcutaneous administration of BPA (0.025, 0.25, or 25 μg/kg BW/day) also disrupted global metabolism including variance in glucose, pyruvate, some amino acids, and neurotransmitters in perinatally exposed CD-1 mouse pups,[67] indicating that perinatal BPA exposure could disrupt energy metabolism and brain function in mice. Samuel et al. reviewed the vivo studies concerning the pathophysiology of obesity after BPA exposure in animals, and concluded that BPA can lead to metabolic and gut microbiota disorders which are directly related with obesity.[68]

1.5.2.2 Effects on neurodevelopment:

The effects of BPA on neurodevelopment have been evaluated in different animal models. Developmental exposure to BPA (2 and 200 μg/kg BW/day by gavage) increased anxious behaviour of female mice in a dose-dependent fashion (male mice were not tested).[69] Cox et al. also found that gestational exposure to BPA affected social behaviour, such as spent more time sniffing a novel adult,

and increased anxiety in juvenile mice; however, the effects were modified if the mice were reared by dams who did not exposed to BPA during pregnancy.[70] In contrast, perinatal exposure of BPA (40 and 400 μg/kg BW/day by gavage) reduced anxiety and motivation to explore in male rats, and depressed the motor activity and motivation to explore in female rats.[71] In addition, the effects from the prolonged low dosage were comparable with those in a higher dosage group during a shorter steroid-sensitive period,[71] which raises concern for the health risk of prolonged exposure to low levels of BPA in humans.

Treatment of embryonic zebrafish with low concentrations of BPA (0.0068 μM) in the embryo medium (equal to a dosage of 0.082 μg/kg in embryo), equivalent to 1,000-fold lower than the acceptable human daily exposure from different sources such as Health Canada and the EFSA, resulted in 180% increases in neuronal birth (neurogenesis) within the hypothalamus, a highly conserved brain region involved in hyperactivity.[72] In addition to rodent and fish models, continuous subcutaneous BPA administration in a nonhuman primate model, at a dose equal to the reference safe limit (50 μg/kg BW/day) of the U.S. Environmental Protection Agency (EPA), abolished the estradiol-induced spine synapse formation.[73] Prenatal BPA exposure in cynomolgus monkeys (10 μg/kg BW/day via subcutaneously implanted pump) altered behavioural sexual differentiation in male infants during the early suckling period.[74] Other studies, however, did not find that BPA effected anxiety levels, exploratory activity or motor activity in rodents.[75,76] The differences in dosage, sample sizes and concordance across behavioural tasks could be the reasons of the inconsistent results.

1.5.3 Epidemiological studies:

Due to the observed effects in animal studies, epidemiological studies of BPA have mainly focused on the possible effects of perinatal and childhood exposure on development.

1.5.3.1 Effects on adiposity and obesity:

The obesogenic effects of early-life BPA exposure have been evaluated in prospective cohort studies.[77–89] Negative associations between prenatal BPA exposure and birth outcomes including birth weight and gestational length were observed in several studies, and the association is stronger among girls than boys.[79–81] Vafeiadi et al. found that prenatal BPA exposure was negatively associated with body mass index (BMI) and adiposity measures from 1-4 years of age in girls, but the association was positive in boys during this period.[88] In contrast, prenatal and early-childhood BPA exposure has been reported to be positively correlated with BMI, waist circumference, fat mass index, % body fat and body weight growth in childhood from age 2 to 9.[77,85,87–89] Other studies reported no association between prenatal and early-childhood BPA exposure and birth outcomes, BMI or fat mass in childhood.[78,82–86]

Several cohort studies evaluated the effects of prenatal and childhood BPA exposure on adiposity. Harley et al. evaluated the association between prenatal and childhood BPA exposure (at 5 and 9 years of age) and BMI, waist circumference, percent of body fat, and obesity at the ages of 5 and 9.[87] They found that prenatal BPA exposure was negatively associated with BMI at 9 years of age in girls but not boys, while BPA concentrations at 9 years were positively associated with BMI and other adiposity measures at 9 years in both girls and boys. No association was found between urinary BPA concentrations at 5 years of age and any anthropometric parameters at 5 or 9 years. Similar equivocal obesogenic effects related to prenatal and childhood BPA exposure, and gender-related obesogenic effects were also reported in another cohort study in Greece.[88] Prenatal BPA exposure was negatively associated with BMI and adiposity measures in girls, whereas a positive association was found in boys.[88] BPA exposure at 4 years of age was positively associated with BMI z-score, waist circumference and sum of skinfold thickness in all children.[88] In summary, the available epidemiological evidence in obesogenic effects of early-life BPA exposure is ambiguous. As diet is an important exposure source of BPA in humans [2,30–32] and dietary pattern is related to adiposity, confounding factors may be responsible for some observed associations between BPA exposure and adiposity.

1.5.3.2 Effects on neurodevelopment:

As reviewed by Ejaredar et al., associations between BPA exposure and children's behavior were found in epidemiological studies, but reported sex-specific effects differed between studies.[90] For example, based on data from a longitudinal birth cohort study in the USA, Harley et al. observed that prenatal BPA exposure levels were associated with increased internalizing problems (i.e. anxiety and depression) in boys but not in girls (n=292, 98.6% were Latino) at the age of 7 years.[91] After adjusting for postnatal BPA exposure in children in the Columbia Center for Children's Environmental Health New York City cohort, Perera et al. also found that high prenatal BPA exposure was associated with significantly higher scores on the Child Behaviour Checklist (CBCL) (i.e. more problems) on Emotionally Reactive and Aggressive Behaviour syndromes among boys between 3 and 5 years of age (n=198). Whereas among girls, higher exposure was associated with lower scores (i.e. fewer reported behaviour problems) on all syndrome scales, reaching statistical significance for less Anxious/Depressed and Aggressive Behaviour.[92] In contrast, based on the data from a prospective birth cohort in Cincinnati, Ohio (USA), Braun et al. found that prenatal BPA exposure was associated with externalizing scores evaluated at 2 years of age using the 2nd edition Behavioural Assessment System for Children (n=249), but significant association was only observed among girls.[93] Using data from the same cohort, Braun et

al. also reported that the positive associations between gestational BPA exposure and anxious and depressed behaviour were stronger among girls than boys at 3 years of age (n=244).[94] Compared with 26-week and birth concentrations, BPA concentrations collected at approximately 16 weeks of gestation were more strongly associated with externalizing scores among all children, suggesting that the early period of pregnancy is a more vulnerable time for BPA exposure.[93]

Associations between childhood BPA exposure and neurobehaviour were also evaluated in epidemiological studies.[90] Using data from a longitudinal birth cohort study in the USA, Harley et al. reported that childhood exposure to BPA was positively associated with externalizing behaviours in girls at age of 7, and with internalizing behaviours, inattention and hyperactive behaviours in both boys and girls at age of 7. [91] In a prospective cohort study in New York City (USA), Roen et al. found that high postnatal BPA exposure was associated with increased internalizing and externalizing behaviours in girls but decreased behaviour symptoms in boys at 7 to 9 years of age.[95] Other studies, however, have reported that childhood BPA exposure was only negatively associated with Emotionally Reactive scores (i.e. one of seven CBCL syndrome scores) [92] or no significant association between childhood BPA exposure and child behaviours.[94]

1.5.3.3 Potential confounding and interacting factors in epidemiological studies of BPA

Children's sex, family income, maternal education level, maternal age, gestational age, smoking status and ethnicity were commonly adjusted as covariates in statistical models for evaluating the effects of BPA on neurodevelopment of children.[92,96]

Exposure to BPA is mainly through the diet,[30–32] which could also be an important exposure source of other neurotoxicants. For example, high levels of heavy metals are detected in canned fish,[97] which is also regarded as an important exposure source of BPA.[33,98] The general population is widely exposed to these heavy metals, such as lead and total mercury,[99] and these are already established as developmental neurotoxicants based on animal and epidemiological studies.[99] Thus, co-exposure of BPA and classic neurotoxicants should be considered when investigating the association between BPA exposure and neurodevelopment of children.

The maternal dietary supply of micronutrients during pregnancy has a well-established influence on fetal growth and has the potential to impact future health conditions including behavioural problems and cognitive function.[100,101] The supply of micronutrients such as iron, zinc, copper, selenium, vitamin B12, choline, folate and long chain polyunsaturated fatty acids are essential for brain development and function.[102–104] For example, children with chronic iron deficiency in infancy had higher developmental

and behavioural risk, even after 10 years of iron treatment.[105] Vitamin B12 is an essential element in the synthesis of some neurotransmitters that are required for neurotransmission.[103] Choline is the precursor of the acetylcholine, which is an important neurotransmitter.[104] Selenium could affect brain development through thyroid hormone metabolism.[106] Docosahexaenoic acid (DHA) and arachidonic acid (AA) are long-chain polyunsaturated fatty acids that have high concentration in the brain and influence the neuron's plasma membrane, synaptogenesis, and myelination.[102] Folate supplementation is recommended for women prior to pregnancy to reduce the risk of neural tube defects in the baby.[107]

To our knowledge, no epidemiological studies examining the neurodevelopmental effects of BPA exposure have considered maternal nutrient status during pregnancy, nor co-exposure to other classic neurotoxicants. In our epidemiological study of BPA exposure in Chapter 5, I therefore considered measurements of prenatal exposure to four heavy metals (total mercury, lead, cadmium and arsenic) which are established as developmental neurotoxicants,[99] and I furthermore utilized data on maternal status of iron, zinc, copper, selenium, manganese, vitamin B12, choline, folate, DHA and AA during pregnancy.

As introduced above, the interaction between BPA exposure and child gender on behavioural problems of children were observe in previous epidemiology studies.[92,94,95,108] This may indicate that BPA's effects on children's behaviour is linked to its weak estrogenic effects,[109] which would presumably affect boys differently than girls. Besides endocrine-related effects, BPA's ability to disrupt DNA methylation at certain gene loci has been demonstrated in animal models.[110–113] Wolstenholme et al. reviewed the *vitro* and *vivo* studies of epigenetic, brain and behavioural effects of BPA, and concluded that BPA's effects on the DNA methylation might be an important mechanism linked to its effects on the brain and behaviour.[109] Interestingly, the epigenetic effects of BPA on DNA methylation were found to be counteracted by supplementation of nutrients involved in DNA methylation in animal studies.[110,111] This led us to a hypothesis that dietary intake of these nutrients might mitigate the effects of BPA on DNA methylation, thus blocking any adverse effects on neurodevelopment. For this reason, in Chapter 5 of this thesis, the status of nutrients involved in DNA methylation (i.e. folate, vitamin B12, choline) was examined separately in the statistical modeling.

1.6 Pharmacokinetics of BPA:

1.6.1 Pharmacokinetics of BPA after oral exposure:

After ingestion, free BPA – regarded as the toxic form of BPA – is efficiently metabolized by first-pass metabolism in the intestine and/or liver to nontoxic metabolites which are quickly eliminated through urine in both animals and humans.[35,36,114] More than 90% of orally ingested BPA is eliminated via urine within 24 hrs.[35,36] The percentage of total BPA present as free (i.e. unconjugated) BPA was less than 1% in urine and serum samples collected after oral exposure.[36] The main metabolite was BPA-glucuronide (Bisphenol A mono-beta-D-glucuronide), accounting for 87±6.9% (average ± standard deviation) of total BPA in urine and 62±12% of total BPA in serum.[36] BPA-sulfate (Bisphenol A sulfate) is another important BPA metabolite, accounting for 3±2.3% of total BPA in urine and 23±7% of total BPA in serum following oral exposure.[36] Although only in one report, low concentrations of BPA-disulfate (Bisphenol A bissulfate) were also detected in human urine and serum of background humans.[115] The structures of these BPA metabolites are shown in Table 1-1. Elimination has been shown to be biphasic, with first phase plasma elimination half-life from the human study of Völkel et al. (2002) being approximately 1.5 hrs, and the terminal phase blood elimination half-life being reported as 3.4 hrs.[35] The terminal phase blood elimination half-life was estimated to be 6.4 hrs in another pharmacokinetic study of oral BPA exposure in humans.[36]

1.6.2 Pharmacokinetics of BPA after dermal exposure:

The pharmacokinetics of BPA in humans following dermal exposure are less clear, as there were no controlled studies in humans prior to this thesis work. *In vivo* animal dermal absorption studies and *in vitro* skin penetration experiments have been used to extrapolate to humans, but with varying results. For example, one study concluded that dermal absorption of BPA (dosage: 1.8 μg BPA/cm^2) was likely negligible, as only 8.6% of the dose penetrated through human skin after 24 hrs incubation *in vitro*.[116] Another study measured the percutaneous BPA absorption *in vitro* on human skin using a higher dose (200 μg BPA/cm^2) and *in vitro* and *vivo* in the rat.[117] The penetration flux of BPA in human skin (0.12 μg/cm^2/hr) estimated from their results was more than five times of that in the above study (0.022 μg/cm^2/hr). Based on their results, they estimated that 1hr occupational exposure over 2,000 cm^2 (forearms and hands) may lead to a BPA absorption of 4 μg/kg BW/day, and the dermal BPA exposure from thermal paper may be toxicologically relevant.[117] Inconsistent results in metabolism efficiency of BPA were also observed *in vitro* human skin studies. For example, one *in vitro* study in human skin concluded that absorbed BPA was not biotransformed (i.e. <3%),[117] while another study with viable human skin demonstrated 27% conjugation of BPA to the glucuronide and sulfate.[118] As discussed by Demierre et al.,[116] the species, the dose and penetration times were important variables in these

experiments. A current knowledge gap, therefore, is the lack of *in vivo* human data on dermal BPA absorption and subsequent pharmacokinetics following a realistic dermal exposure scenario. Teeguarden et al. recently cited an unpublished dermal BPA exposure study using isotope labelled BPA,[119] but to our knowledge this study is not yet publicly available. Chapter 3 compares pharmacokinetics of BPA in human volunteers following dietary and dermal exposure using isotope-labeled BPA.

1.7 Biomonitoring of BPA in human samples:

1.7.1 Biomarkers of BPA exposure

1.7.1.1 Total BPA in urine and blood:

Human biomonitoring of BPA exposure has generally relied on detection of "total BPA" in urine; that is, the sum concentration of all metabolites and free-BPA following an enzymatic digestion. There are at least three reasons to choose urinary total BPA as the biomarker of BPA exposure: 1) as BPA is quickly and completely eliminated through urine after exposure, [35,36,114] the BPA concentrations in urine are much higher than those in other biological samples; [120] 2) urine collection is non-invasive, which is more welcome for human studies; and 3) by quantifying total BPA in 24 hr urine samples, the daily intake of BPA can be easily calculated. Total BPA was detectable in 90% of urine samples (>0.4 ng/mL) with a GM of 1.5 ng/mL in the 2011-2012 NHANES survey.[29] According to national surveys in Canada (2012-2013), approximately 92% of the general population have detectable urinary concentration of BPA (>0.2 ng/mL) with GM of 1.1 ng/mL.[28]

As BPA concentrations are suggested to be 10- to 100-fold lower in blood compared to urine samples, [35,36,121,122] fewer studies have evaluated the BPA concentrations in human blood. Zhange et al. analyzed total BPA in paired whole blood and urine samples (n=50 pairs) and found a significant correlation between creatinine-adjusted urinary total BPA concentration and blood total BPA concentration.[122] The concentrations measured in urine were approximately an order of magnitude higher than the concentrations found in blood.[122] Table 1-2 reviews studies on total BPA in paired maternal and cord serum. Total BPA concentrations in paired maternal and cord serum were significantly correlated.[123,124] Due to common BPA contamination in blood sampling materials, the measurements of total BPA in human blood are often questioned.[125,126]

1.7.1.2 BPA metabolites in urine and blood:

As introduced previously, more than 90% of total BPA in urine and more than 99% of the total BPA in blood are explained by conjugated forms of BPA after oral exposure.[36] However, BPA exposure from non-dietary pathways, such as via dermal exposure, would bypass first-pass metabolism and might lead to slower or lower detoxification effeciency (i.e. higher proportion of free BPA may be in systemic circulation after dermal exposure). Thus, separate analysis of BPA metabolites and free BPA in biofluids might be valuable for distinguishing BPA exposure from different exposure pathways.

The quantitation of BPA-glucuronide and free BPA instead of total BPA was first described by Völkel et al.[127] A national survey by Health Canada showed that BPA-glucuronide was the predominant form of BPA in 1,890 first-trimester urine samples.[128] The GM of the ratio of BPA-glucuronide to total BPA was 90.27%, while the GM ratio of free BPA to total BPA was only 1.01%.[128] Gerona et al. directly measured free BPA, BPA-glucuronide and BPA-sulfate in maternal urine collected in the 2nd trimester.[129] On average, total BPA consisted of 71% BPA-glucuronide, 15% BPA-sulfate and 14% free BPA. Moreover, the proportion of BPA-sulfate increased and the proportion of free BPA decreased with increasing total BPA.[129]

Previous reports of successful BPA metabolite analysis in serum of the general population are effectively limited to only one study,[115] in which BPA-glucuronide (46% of total BPA) was somewhat higher than BPA-disulfate (34%), but BPA-(mono)sulfate was not measured. Teeguarden et al. attempted to monitored BPA-glucuronide and BPA-sulfate in serum of pregnant women, but only a few samples had concentrations higher than the LODs of their method (0.045-0.35 ng/mL for BPA; 0.075 ng/mL for BPA-glucuronide; 0.031 ng/mL for BPA-sulfate).[119] Gerona et al. found higher concentrations of BPA-sulfate (GM: 0.32 ng/mL, range <0.025 - 12.65 ng/mL) than BPA-glucuronide (GM: 0.14 ng/mL, range <0.05 - 5.41 ng/mL) in midgestation cord serum.[130] This result is in contrast to published oral exposure studies in adults, in which BPA-glucuronide concentrations were significantly higher than BPA-sulfate in serum after controlled oral BPA administration.[35,36] This may be explained by the unique fetal biotransformation system. Biotransformation capacity in the human fetus is not mature, and uridine 5'-diphospho-glucuronosyltransferase activity is limited for neonates,[131] whereas the ontogeny of sulfotransferases is earlier and is believed to be the main conjugation pathway for BPA before birth.[131,132] The known activity of β-glucuronidase in the placenta or fetal liver, in tandem with relatively high sulfotransferase activity in fetus, may therefore be responsible for the observed metabolite profiles in cord serum.[132]

1.7.2 Difficulties in biomonitoring of BPA:

1.7.2.1 Background contamination of BPA from sample collection and analysis:

A major difficulty in biomonitoring of BPA is contamination of free BPA during sample collection, storage and analysis.[120,125,133,134] The contamination of BPA during biomonitoring analysis could come from solvents and reagents, the analytical instruments, the laboratory environment and/or even the analyst.[134] Longnecker et al. found that the concentration of BPA in urine could be affected by specimen collection, storage and handling.[133]

Due to the expected low BPA concentrations in human blood, BPA contamination could bias the true exposures in blood samples.[120] Gyllenhammar et al. found that blood sampling materials were leaching BPA, and that BPA contamination probably occurred randomly during sample collection, handling, pooling and processing.[126] Nevertheless, the levels of free BPA in serum from primiparous women were generally less than the limit of quantification (LOQ, 0.2 ng/mL) when contamination was minimized.[126] Teeguarden et al. reviewed the methods used for detecting serum concentrations of BPA in humans and concluded that reported BPA in human serum is questionable and is too low to cause an estrogenic effect.[125]

1.7.2.2 Temporal variability of BPA concentrations in human samples:

Owing in part to dietary intake and the short elimination half-life,[35,36] there is known within-person variability of BPA concentrations (after creatinine adjustment) in spot, first morning and 24 hrs urine samples.[135] Arakawa et al. measured total urinary BPA concentration in 24 hr urine samples from 5 subjects for 5 consecutive days and found a large intra-individual variation (91%) over 5 days for the daily excretion of urinary BPA.[136] Ye et al. observed considerable within-day variance (70%) for spot urine samples, which outweighed the between-person (9%) and between-day and within-person (21%) variances.[135] Nevertheless, they concluded that when the population investigated is sufficiently large and samples are randomly collected, relative to meal ingestion times and bladder emptying times, spot urine samples may adequately reflect the average exposure of BPA in a studied population.[135] Fisher et al. evaluated the daily and across pregnancy variability of urinary BPA and found low reproducibility of BPA throughout pregnancy and into the postpartum period, but much higher reproducibility within a day.[137] Urinary concentration of BPA in the evening (18:00-23:59) was highest and the GM concentration was relatively low in the morning (8:00-11:59).[137] This daily variance could be explained by the exposure time (i.e. meal time) and the short biological half-life of BPA (<6hr) from oral exposure.[35,36] However,

contrasting this result was that urinary BPA concentration was significantly higher in samples collected before 7:00 AM in pregnant women in France.[138]

1.8 Placental transfer of BPA:

Pregnancy is a critical window of environmental exposure for mothers due to rapid and sensitive developmental processes in the fetus. Moreover, fetal biotransformation pathways are immature [131,132] and could lead to different BPA metabolite patterns in both the fetus and the mother. As BPA is a small chemical (228 g/mol) with moderate lipophilicity (log K_{ow} of 3.4) and not ionized at physiological pH levels, unconjugated free BPA could easily transfer through the placenta in a similar manner as ethanol and antipyrine.[139–141] The human placenta has only limited BPA conjugation ability, whereby only 3.2% of BPA that transferred was conjugated to metabolites in human placenta.[141] For conjugated BPA, which is larger and easily ionized at physiological pH, selective transfer processes through protein channels are involved in its placental transfer.[142] However, the transfer efficiency of conjugated BPA is even lower than of free BPA.[140,142] What is more, after uterine perfusion of BPA-glucuronide, deconjugated free BPA became detectable in the fetus and amniotic fluid,[142] suggesting that fetal tissues have the ability to deconjugate the BPA-glucuronide. A study in sheep showed that on average 67% of BPA entering the fetal compartment was rapidly eliminated through fetal to maternal clearance (half-life 20 min), but that 24% remained trapped as BPA-glucuronide in fetal circulation.[143] This conjugation-deconjugation cycling of BPA was responsible for a 43% increase of overall fetal exposure to free BPA in the sheep study and led to low but sustained free BPA exposure in the fetus with a prolonged hydrolysis-dependent plasma terminal half-life (>100 hrs for free BPA).[143] The slower clearance of conjugated BPA from the fetus relative to maternal serum was also observed in other animal studies of sheep and rhesus monkey.[144,145]

Due to the relatively low BPA concentrations in blood samples, its short biological life and difficulties in quality control during sampling, the placental transfer of BPA in humans is not well studied. The published studies of total BPA in paired maternal and cord serum are listed in Table 1-2. These studies show that total BPA was detectable in cord serum from North America, Europe and Asia. The total BPA concentrations in cord blood were relatively lower than in paired maternal blood (Table 1-2), which could be explained by lower placental transfer of conjugated BPA,[140,142] or by fetal excretion of these xenobiotics to amniotic fluid. The latter explanation is partly supported by a pregnant rhesus monkey BPA exposure study, wherein higher concentrations of free and conjugated BPA accumulated in amniotic fluid during the pregnancy.[145] However, as I introduced previously, the detection of total BPA in

human blood is questionable and the true levels of total BPA in human blood could be easily obscured by BPA contamination during sample collection, processing, storage and analysis. Thus, direct monitoring of BPA metabolites in paired maternal and cord serum may provide valuable information for risk assessment. To address this issue, the study introduced in Chapter 2, which directly measured the three typical BPA metabolites in paired maternal and cord serum was conducted.

1.9 BPA alternatives:

Owing to increased scientific scrutiny of BPA, and existing bans on its inclusion in certain consumer products, BPA-alternative chemicals have increased in use.[146,147] Products labeled with "BPA free" might contain these BPA alternatives. Table 1-1 lists the structures of 4 important BPA alternatives. Based on available data, these alternatives have similar molecular sizes and structures compared to BPA, and offer no obvious advantages in terms of their endocrine-disrupting activities,[148,149] aquatic toxicity,[1] persistence[1,150] or bioaccumulation potential.[1]

Bisphenol S (BPS; bis(4-hydroxyophenyl) sulfone) is one such BPA alternative that has already been widely used in a variety of industrial applications, such as a wash fastening agent in cleaning products, an electroplating solvent, and a constituent of phenolic resin.[151] It is also used as a developer in thermal paper and frequently detectable in store receipts, on currency and in other paper products. [39,43] Biomonitoring has shown that BPS was detected in 81% of urine samples from China, USA, and in 6 other Asian countries.[152]

Bisphenol F (BPF; bis(4-hydroxyphenyl)methane), an important BPA alternative, is used to make epoxy resins and coatings for systems such as tank and pipe linings, industrial floors, road and bridge deck toppings and structural adhesives.[153] BPF has been detected in many consumable products such as personal care products [147] and foodstuff.[154] The detection frequency and concentration of BPF were even higher than BPS in 616 urine samples from U.S. adults between 2000 and 2014.[155] Although average concentrations of BPF were still lower than BPA, the 95th percentile concentration of BPF was often comparable or even higher than BPA.[155]

Bisphenol AF (BPAF; 4,4'-(hexafluoroisopropylidene) diphenol) was detected in river water, sediment,[19] indoor dust [25] and detectable with low frequency (<3%) in human urine.[155] Bisphenol B (BPB; 2,2-bis(4-hydroxyphenyl)butane) is a BPA alternative and has been detected in foodstuffs with low detection frequency,[154] and was detected in 27.6% of serum samples (>0.18 ng/mL) from women with

endometriosis.[156]

Compared to BPA, fewer research studies have evaluated the exposure levels and exposure sources of these BPA alternatives. No study has evaluated human exposure levels of these BPA alternatives in Canada. In addition, to our knowledge, there are currently no data on human *in utero* exposure to any BPA alternative chemicals and subsequent adverse effects in children. Therefore, these BPA alternatives are included in several chapters of this thesis.

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Table 1-1 Full name and chemical structure of BPA, 3 BPA metabolites and 3 BPA alternatives.

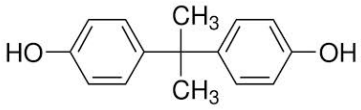
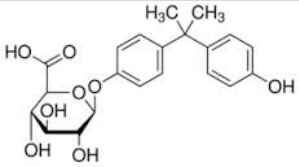
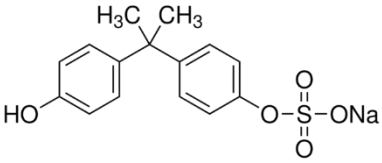
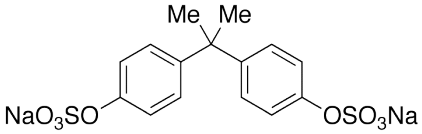
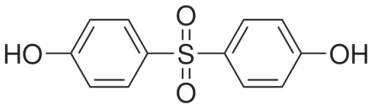
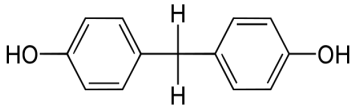
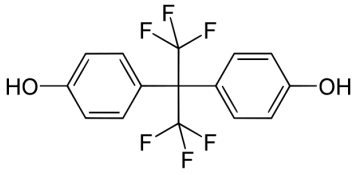
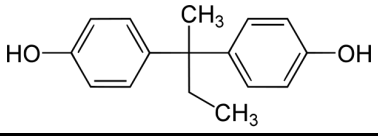
Compounds	Full name	Chemical structure
BPA	Bisphenol A (4,4'-propane-2,2-diyldiphenol)	
BPA metabolites		
BPA-glucuronide	Bisphenol A mono-beta-D-glucuronide	
BPA-sulfate	Bisphenol A sulfate (sodium salt)	
BPA-disulfate	Bisphenol A bissulfate (disodium salt)	
BPA alternatives		
BPS	Bisphenol S (bis(4-hydroxyphenyl)sulfone)	
BPF	bis(4-hydroxyphenyl)methane	
BPAF	Bisphenol AF (4,4'-(Hexafluoroisopropylidene)diphenol)	
BPB	Bisphenol B (2,2-Bis(4-hydroxyphenyl)butane)	

Table 1-2 Concentrations of total BPA (ng/mL) in paired fetal and maternal serum in other published studies.

Reference	Year, location	Sampling time of maternal blood	Fetal mean ± SD (n, n>LOD)	Maternal mean ± SD (n, n>LOD)
[158]	2008-2009, Korea	third trimester	<0.6 (25, 2)	0.7 ± 0.1 (26, 7)
[123]	Korea	full term	1.13 ± 1.43 (300, 120)	9.04 ± 14.03 (300, 252)
[159] [a]	2000-2001, Germany	32-41 weeks of gestation	2.9 ± 2.5 (37, 37)	4.4 ± 3.9 (37, 37)
[160]	Canada	before delivery	2.17 (0.12-7.75) [b] (35, 35)	3.0(0.04-24.2) [b] (35, 35)
[124]	Canada	before delivery	1.23 ± 1.04 (61, 58)	1.36 ± 1.18 (61, 59)
[161]	USA	≥37 weeks of gestation	1.3 (0.14-153.5) [b] (27, 26)	14.1 (0.14-25.6) [b] (27, 22)

[a] Plasma was used for this study.
[b] Median (range).

2 Bisphenol A Metabolites and Bisphenol S in Paired Maternal and Cord Serum ①

2.1 Introduction

BPA is a weak estrogenic chemical used for over 40 years for manufacturing polycarbonate plastic and epoxy resins, including for applications as a resin lining of food and beverage cans, in hard plastic food containers, and in medical devices and toys.[1] Animal toxicology studies have shown effects of perinatal BPA exposure on energy metabolism, brain function, and on development and behaviour at low-doses,[2–6] but as reviewed by Teeguarden et al. these "low dose" animal studies still represent higher exposure than experienced by humans.[7]

According to national surveys in Canada (2012-2013) and the United States (2003-2012), approximately 90% of the general population had detectable urinary concentration of BPA (i.e. >0.2 or >0.4 ng total BPA/mL).[8,9] In China, BPA was detected in 96% of urine samples (>0.10 ng total BPA/mL) from women collected in 2010 (n=23).[10] Current human evidence shows that gestational BPA exposure is associated with anxious, depressive and hyperactive behaviours in children, with the effects differing by gender.[11–13] However, results in such human studies are inconsistent. For example, Harley et al. observed that prenatal BPA exposure levels were associated with increased internalizing problems (i.e. anxiety and depression) in boys but not in girls (n=292), at age of 7.[14] Using the same behaviour assessment method, Braun et al. also reported that gestational BPA exposure was positively associated with anxious and depressed behaviour, at 3 years of age (n=244), but the associations were stronger among girls than boys.[11]

Diet is currently regarded as the major human exposure pathway to BPA,[15–17] but exposure from dermal contact and dust can also occur.[18,19] When exposure to BPA occurs orally, human studies have shown that BPA can be metabolized to polar conjugates (>99%) and rapidly cleared through urine (half-life<6 hrs).[20,21] The proportion of free BPA (i.e. the most toxic form of BPA [22]) comprises less than 1% of total BPA in blood and urine following oral exposure.[20,21] The polar BPA metabolites (mainly BPA-glucuronide and BPA-sulfate) have previously been characterized as non-toxic relative to parent BPA with regards to endocrine disrupting[23–25] and cytotoxic effects.[26] Nevertheless, endocrine disrupting activity of BPA metabolites has been observed in a rat prolactinoma cell line,[27] and obesogenic effects of the metabolites have been noted in human and murine preadipocytes.[28]

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① A version of this chapter has been published as *Environ. Sci. Technol.* **2017**, 51(4), 2456-2463.

Human biomonitoring of BPA exposure has generally relied on detection of "total BPA" in urine; that is, the sum concentration of all metabolites and free-BPA following an enzymatic digestion. The quantitation of BPA-glucuronide and free BPA instead of total BPA was first described by Völkel et al.[29] For pregnant women and the developing fetus, it is desirable to understand the circulating maternal and fetal blood concentrations of BPA and its metabolites, not just total BPA. As noted above, pregnancy is a critical window of exposure due to developmental processes in the fetus. Fetal biotransformation pathways are immature[22,30] and could lead to different BPA metabolite patterns in both the fetus and the mother. Moreover, animal studies have shown a slower clearance of conjugated BPA from the fetus relative to maternal serum.[31,32] BPA-glucuronide administered to fetal sheep was not only cleared slower, but also interconverted to free BPA and led to a low but sustained free BPA exposure in the fetus (half-life>100 hrs).[32] Thus, direct monitoring of BPA metabolites in paired maternal and cord serum may provide valuable information for risk assessment.

A major difficulty in monitoring BPA in human blood is the possible contamination of free BPA during sample collection, including from typical venipuncture needles and tubing.[33] Thus, monitoring specifically for BPA metabolites in serum is much more reliable than monitoring total BPA. Given that most BPA is present as metabolites in blood following oral exposure,[20,21] and oral exposure is considered the main exposure pathway for BPA,[15–17] BPA metabolite concentrations in blood should be reliable measures of BPA exposure.

Owing to increased scientific scrutiny of BPA, and existing bans in certain consumer products, BPA-alternative chemicals have increased in use.[42,43] These alternatives have similar molecular sizes and structures compared to BPA, and offer no obvious advantages in terms of their endocrine-disrupting activities,[36,37] aquatic toxicity,[38] persistence[38,39] or bioaccumulation potential.[38] BPS is one such BPA alternative that has already been detected in 81% of urine samples from China, the United States, and in 6 other Asian countries.[40] BPAF was detected in river water, sediment,[41] indoor dust [42] and detected with low detection frequency (<3%) in human urine.[43] BPB was a BPA alternative detected in foodstuff with low detection frequency.[44] However, to our knowledge there are currently no data on human *in utero* exposure to any BPA alternative chemicals.

In the present study, three BPA metabolites and three BPA alternatives were analyzed in 61 paired samples of maternal and cord sera collected from participants in two cities in China. Results are discussed with respect to placental transfer, and unique metabolite profiles observed during pregnancy.

2.2 Experimental section

I conducted all of the experiment work and data analysis.

2.2.1 Study population.

The study protocol was reviewed and approved by the ethics committee of the China National Center for Food Safety Risk Assessment, and by the Health Research Ethics Board of the University of Alberta (Study ID: Pro00038394). Between Nov. 2012 and Mar. 2013, 61 pairs of maternal and cord blood samples were collected from recruited pregnant women and their newborns in Beijing (31 pairs) and Shijiazhuang (30 pairs), China.[②] All participating women were informed of the nature and purpose of the study and informed consent was received prior to inclusion in the study. The mean age of participating women was 29, ranging from 24 to 37. All women were healthy and gave birth to a single baby without genetic disorders, and the sex ratio (male/female) of newborns was 27/34. A limitation of the present study was that no information was available on use of medications, intravenous infusions, a time in the hospital. However, total BPA concentrations in the present study are relatively low compared to other studies of total BPA in paired maternal and cord serum (Table 1-2), suggesting that the current participants unlikely had significant BPA exposure in the hospital.

2.2.2 Sample collection and contamination tests. [③]

China, especially in big cities, pregnant women often stay in hospital for several days before delivery, providing this project an opportunity to collect maternal blood samples 1-2 days prior to cord blood collection under controlled conditions. All maternal blood samples were collected in the morning before breakfast, and cord blood samples were collected ex utero immediately after delivery. After centrifugation, serum was transferred to 1.5mL cryogenic vials and stored at -20^{o}C. Sera samples were transported on dry ice, within one week of collection, to the China National Center for Food Safety Risk Assessment where the samples were stored at -80 °C until analysis.

Blood collection kits consisting of stainless steel needles and polyvinyl chloride tubing were used for blood collection. No BPA-metabolite or BPA alternative was detected above detection limits in blank blood collection kits (n=20), serum tubes or in cryogenic storage vials used for sample collection and

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② Dr. Yoning Wu, Dr. Yunfeng Zhao and Dr. Jingguang Li in China National Center for Food Safety Risk Assessment were responsible for the sample collection.

③ Dr. Lin Yang in China National Center for Food Safety Risk Assessment contributed to the blank test of sampling materials.

storage. I also did not detect any free BPA in the blank blood collection kits examined. Free and total BPA concentrations are listed in Table 2-1 but are not discussed further in this paper due to expert opinion that contamination of blood samples from sampling is a common problem which biases results.[33]

2.2.3 Standards and reagents.

BPA-glucuronide (95%), isotopically labeled bisphenol A mono-β-D-glucuronide ($^{13}C_{12}$-BPA-glucuronide; 95%), BPA-sulfate (95%) and BPS (98%) were purchased from Sigma-Aldrich (St. Louis, MO). Bisphenol A bissulfate disodium salt (BPA-bissulfate) was obtained from Toronto Research Chemicals (Toronto, ON, Canada). BPAF (>98%) and BPB (>98%) were purchased from TCI America (Portland, OR). BPA and $^{13}C_{12}$-BPA (99%) was acquired from Cambridge Isotope Laboratories (Andover, MA). β-glucuronidase enzyme from *Helix pomatia* (≥100,000 units/mL β-glucuronidase; ≤7500 units/mL sulfatase) was purchased from Sigma-Aldrich. LC grade water was acquired from Fisher Scientific (Fair Lawn, NJ) and methanol was from J&K Scientific (Beijing, China). LC grade ammonium acetate was obtained from Dikma Pure (Richmond Hill, GA). Analytical grade formic acid (98%) and ammonium hydroxide (NH_4OH, 25% in water) was supplied by Xin Guang (Beijing, China).

2.2.4 Sample preparation.④

Each serum sample was defrosted, vortexed, and two separate aliquots of 0.5mL were subsampled. For analysis of total BPA, total BPS, total BPAF and total BPB, one of these aliquots was spiked with 1 mL of 1 M ammonium acetate buffer containing 5μL β-glucuronidase enzyme and 10μL of 0.5ng $^{13}C_{12}$-BPA-glucuronide (used to monitor the effectiveness of the in vitro deconjugation reaction). This aliquot was then incubated at 37 °C overnight for deconjugation prior to solid phase extraction (SPE). For analyzing BPA metabolites, free BPA and free BPA alternatives, the other aliquot was spiked with 10 μL of internal standard mixture containing 1 ng $^{13}C_{12}$-BPA and 0.5 ng $^{13}C_{12}$-BPA-glucuronide, equilibrated for 1 hour and subsequently extracted by SPE (i.e. without enzyme digestion).

The SPE method in the present study was developed based on the method of Liao et al.[45] SepPak C18 SPE cartridges (200mg/3cc; Waters, Milford, MA) were first cleaned by addition of 10 mL methanol, and were then conditioned by addition of 5 mL 1% formic acid. Before loading serum sample aliquots to SPE,

④ Dr. Fengji Luo, Dr. Shuming Li in Chaoyang District Center for Disease Control and Prevention provided the lab resources for the sample preparation in China.

2 mL of 2% formic acid in water was added to each serum sample. After loading of serum, the cartridges were washed with 2 mL of 0.5% formic acid in water, and 4 mL 25% methanol in water. Finally, 3 mL of 5% NH_4OH in methanol was used to elute target analytes, and this was concentrated under nitrogen gas to 0.5 mL for instrumental analysis.

2.2.5 Instrumental analysis.

BPA, BPA metabolites and BPA alternatives were determined using ultra-performance-liquid-chromatography coupled to a triple quadrupole mass spectrometer (Xevo TQ-S, Water, Milford, USA) operating in negative electrospray ionization mode with multiple-reaction-monitoring (MRM, Table 2-2 for transitions). For each extract, 10 µL was injected to a 2.1 × 50 mm BEH C18 column (1.7 µm, Waters, USA). The mobile phases were: (A) 2 mM aqueous ammonium acetate and (B) 100% methanol. Flow rate was 0.2 mL/min, and the gradient elution program was as follows: initial condition 20% B, 0.5 – 5 min ramped to 100% B, hold until 8 min, returning to initial condition by 8.1 min, and re-equilibration until 12 min.

2.2.6 Quality assurance and quality control.

The stability of BPA metabolites was tested by analysis of free BPA in blank calf serum spiked with 1 ng/mL of BPA-glucuronide, BPA-sulfate and BPA-bissulfate. After storage at ambient temperature for 48 hrs, the levels of metabolites were comparable with that in spiked samples stored at -20 C°, and no free BPA was detected, demonstrating the stability of BPA metabolites during sample collection or analysis. The stability of BPA metabolites in serum was also proven previously.[45,46]

For quantifying total BPA and total BPA alternatives, enzyme was added to deconjugate any metabolites back to their free forms. The amount of enzyme added in each sample was optimized to be enough to completely digest 5 ng BPA-glucuronide and 5 ng BPA-sulfate (which is higher than concentrations detected in any sample). The $^{13}C_{12}$-BPA-glucuronide added in serum aliquots with enzyme digestion was completely digested to $^{13}C_{12}$-BPA, which was then used as an internal standard to quantify total BPA and total BPA alternatives (BPS, BPAF, BPB). No BPA metabolites were detectable in samples after enzyme digestion. In serum aliquots analyzed without enzyme digestion, $^{13}C_{12}$-BPA (added directly) was used to quantify free BPA and free BPA alternatives, and $^{13}C_{12}$-BPA-glucuronide was used to quantify each of the three BPA metabolites (BPA-glucuronide, BPA-sulfate, BPA-bissulfate). The reliability of BPA metabolite concentrations was also tested by mass balance and by correlations of total BPA with sum of free BPA and BPA metabolites (Supporting Information).

A typical chromatogram obtained by analysis of unspiked maternal serum is shown in Figure 2-1. The recoveries of target analytes at three levels ranged from 62-106%, with relative standard deviation (RSD) ranging from 3.0% to 10.1% (Table 2-3). Linearity was evaluated over two orders of magnitude (0.05 ng/mL to 5.00 ng/mL, 6 point curve), and the regression coefficients of the standard curves were always > 0.99.

Pure water/methanol (1:1) was injected before the first sample and between every sample to monitor instrument background, and no instrument background or carryover was ever found. Procedural blanks (LC grade water spiked with internal standards) were extracted and analyzed with every batch of up to 11 samples. A stable and low level of free BPA and free BPAF was observed in the procedural blanks (Table 2-2), which was subtracted in quantification of real samples. The LODs (Table 2-2) were the higher value of either: i) signal-to-noise ratio of 3, determined in sample extracts from spiked calf serum, or ii) the average concentration in procedural blanks plus 3 times the standard deviation of the procedural blanks. The LODs of BPA-glucuronide (0.01 ng/mL) and BPA-sulfate (0.01 ng/mL) in the present study were somewhat lower than in a previous study using liquid extraction (0.08 and 0.03 ng/mL),[47] and two other studies (0.02 - 0.05 ng/mL) that also used SPE and liquid chromatography/tandem mass spectrometry for serum analysis.[45,48] However, our LOD for BPA-bissulfate (0.20 ng/mL) was 10-times higher than in these two former studies (0.02 ng/mL for BPA-bissulfate).

2.2.7 Mass Balance and Correlations of Total BPA with Sum of Free BPA and BPA Metabolites.

To test the accuracy of our method in quantification of BPA metabolites, I examined the mass balance and correlation of total BPA (determined by enzyme digestion) with sum of free BPA and BPA metabolites (detected without enzyme digestion). In maternal serum there was no statistical difference between total BPA concentrations and the sum concentration of free BPA and its two major metabolites (BPA-sulfate and BPA-glucuronide), based on molar concentrations (p=0.44). Furthermore, in maternal serum, total BPA was positively associated with the sum concentration of free BPA and its two metabolites (p<0.01, r=0.48).

In cord serum, only 5 of 61 cord sera had detectable free BPA. Among the 56 cord samples with no detectable free BPA, there was also a significant linear correlation (p<0.01, r=0.78) between total BPA and the sum of the two major metabolites. However, in the remaining 56 samples the total BPA concentration was significantly higher than the sum of the two major metabolites (p=0.02). Based on molar concentrations, I can estimate the mean difference to be 0.31 nmol/L, which was lower than the

LOD of BPA in our method (0.53 nmol/L). This suggested that the difference mainly came from the free BPA below the LOD in these samples.

2.2.8 Data analysis.[⑤]

Data analysis was performed with R (v 3.0.2) and Stata 12.0, and the level of significance for all of statistical tests was $p<0.05$. For calculation of mean and GM, non-detect concentrations were assigned a value equal to the LOD divided by the square root of 2 when detection frequency was greater than 35%. As the detection frequency was lower than 40% for BPA-glucuronide in maternal serum, using single values to substitute these non-detects is known to produce poor estimates.[49] To accommodate censoring, I used the *Kendall's Tau* test to analyze the relationship between BPA metabolites in maternal and cord serum. This test is based on the rank concentration of each analyte, and the model is commonly used in tests for trend with censored data.[50] Molar concentrations (nmol/mL) were used to examine relationships between target analytes in cord and maternal serum. *Wilcoxon Signed Rank* Test was used to compare concentrations of all detected analytes in maternal and cord serum. The differences of analytes concentrations between exposure groups and between infant gender groups were assessed by *Kruskal Wallis Test*.

2.3 Results and discussion

2.3.1 Detection of BPA alternatives.

Among 3 BPA alternatives analyzed, only total BPS was detectable. It was furthermore only detected at low frequency, specifically in 4 of 61 enzyme digested maternal sera, and in 7 of 61 enzyme digested cord sera. No free BPS was ever detected, indicating that BPS was predominantly present in conjugated form. Statistics are not possible on this small dataset, but among the 11 samples with detectable total BPS, 4 of these were for paired maternal and cord sera (i.e. 2 paired samples): individual maternal concentrations were 0.07 and 0.03 ng/mL, while those in paired cord serum were 0.08 and 0.04 ng/mL, respectively, suggesting that higher fetal BPS levels were associated with higher maternal concentrations. To our knowledge, this is the first time that this BPA alternative has been detected in paired cord and maternal serum. Although the BPS exposure was much lower than that of BPA in this population, detection of BPS in cord serum here provides the first evidence that this chemical passes the

[⑤] Dr, Irina Dinu and Dr. Elham K. Moez in University of Alberta assisted with the data analysis.

placenta. Co-exposure of BPA and BPA alternatives should be considered in future toxicology or epidemiology studies.

2.3.2 Detection of BPA metabolites.

BPA-glucuronide and BPA-sulfate were detectable in cord and maternal serum with variable detection frequencies (Table 2-4). No gender difference was observed among cord sera samples for any analyte, thus data from male and female births were combined. BPA-bissulfate was monitored but was not detectable in the present study, likely due to the relatively high LOD (0.20 ng/mL). For example, in a previous report BPA-bissulfate was detectable in serum from 8 of 14 volunteers in Albany, NY, but with a low GM of 0.12 ng/mL;[45] below the LOD for the current work.

In both maternal and cord sera, BPA-sulfate was the major metabolite detected (GM: 0.06 ng/mL and 0.08 ng/mL, respectively), significantly higher than BPA-glucuronide (GM: 0.02 ng/mL and 0.04 ng/mL, respectively) (p<0.01). Teeguarden et al. analyzed for BPA-glucuronide and BPA-sulfate in maternal serum samples (but not in cord serum),[47] however their high detection limits (0.075 ng/mL LOD for BPA-glucuronide, 0.031 ng/mL LOD for BPA-sulfate) resulted in very few detections, thus data cannot be compared. Therefore, I can only compare to one other study by Gerona et al.[48] who analyzed 85 mid-gestation cord sera (but not maternal sera) from women in California. Quantitative data were reported for BPA-sulfate (GM: 0.32 ng/mL, 96%>LOD) and BPA-glucuronide (GM: 0.14 ng/mL, 76%>LOD), thus concentrations in the current Chinese populations are low compared to California. Nevertheless, a similarity to the current study was that BPA-sulfate cord serum concentrations were approximately twice as high as BPA-glucuronide cord serum concentrations. The current study provides the first human data for BPA metabolites in paired maternal and cord serum, and results suggest that the human fetus, and pregnant mother, have a unique exposure to BPA metabolites.

2.3.3 Profile of BPA metabolites in maternal and cord serum.

In maternal and cord sera with quantifiable concentrations of both BPA-sulfate and BPA-glucuronide, BPA-sulfate was higher than BPA-glucuronide in 87% (13 of 15 maternal) and 86% (19 of 22 cord) of samples, respectively. To investigate trends in the metabolite profiles with increasing exposure, data were stratified into 4 groups based on total metabolite (sum of BPA-sulfate and BPA-glucuronide) concentrations: >25th to 50th percentile, >50th to 75th percentile, >75th -95th percentile, and >95th percentile. The GM of BPA-sulfate and BPA-glucuronide in each group was calculated and plotted for maternal and cord serum. As shown in Figure 2-2, increasing total BPA metabolite concentrations were driven primarily by increasing concentrations of BPA-sulfate in both maternal and cord serum.

In maternal serum, BPA-sulfate accounted for up to 98% of conjugated BPA in the most highly exposed participants (95th percentile), compared to 72% of conjugated BPA in the least exposed group (25th-50th percentile group). The effect was even stronger in cord serum, where BPA-sulfate accounted for up to 99% of total BPA metabolites in the most highly exposed participants (95th percentile), compared to only 23% of total BPA metabolites in the least exposed group (25th-50th percentile group). It should be noted that even if the proportion of BPA-glucuronide increased to 77% in the least exposed cord serum group, BPA-glucuronide concentrations in this group were not significantly higher than its concentrations in higher exposed cord serum groups (p=0.30).

Gerona et al. (2016) recently reported a similar trend in maternal urine (2nd trimester) whereby the proportion of BPA-sulfate increased with total BPA levels.[51] Nevertheless, the dominance of BPA-sulfate in the relative profiles here is in contrast to published oral exposure studies in adults, in which BPA-glucuronide concentrations were significantly higher than BPA-sulfate in serum after controlled oral BPA administration.[21,52] Previous reports of BPA metabolite detection in serum of the general population are limited to only one study,[45] in which BPA-glucuronide (46% of the profile) was somewhat higher than BPA-disulfate (34%); BPA-(mono)sulfate was not measured. Teeguarden et al. analyzed for BPA-glucuronide and BPA-sulfate in serum of pregnant women, but relatively high LODs for the method resulted in very few detections.[47] Nevertheless, a possible explanation for the dominant BPA-sulfate concentrations in the present study is the focus here on pregnant women close to delivery. As discussed above, Gerona et al.[48] found higher BPA-sulfate than BPA-glucuronide concentrations in midgestation cord serum. Biotransformation capacity in the human fetus is not mature, and uridine 5'-diphospho-glucuronosyltransferase activity is limited for neonates,[30] whereas the ontogeny of sulfotransferases is earlier and is believed to be the main conjugation pathway for BPA before birth.[22,30] The known activity of β-glucuronidase in the placenta or fetal liver,[22] in tandem with relatively high sulfotransferase activity in fetus may therefore be responsible for the observed metabolite profiles here. Thus, unique biotransformation pathways in the human fetoplacental compartment likely led to the high proportion of BPA-sulfate observed here in cord blood, and moreover may have influenced the maternal serum metabolite profile through placental transfer back to maternal circulation. The fetoplacental deconjugation of BPA metabolites leading to transient levels of free BPA has furthermore been discussed with respect to in utero bioactivation.[53,54] In adult sheep, BPA-sulfate was more than 10 times lower than BPA-glucuronide in plasma after infusion of BPA, whereas BPA-sulfate was only 1-2 times lower than BPA-glucuronide in maternal plasma after infusing BPA-glucuronide to fetal sheep. This provides animal evidence for transplacental transfer of BPA metabolites into maternal circulation.[32]

Nevertheless, genetic differences between Asians and Caucasians has been shown to influence glucuronidation and sulfation for other xenobiotics and cannot be ruled out as a factor for the current Chinese population.[55,56] Another possible explanation for the unexpected serum metabolite profiles here is non-dietary BPA exposure routes, such as dermal exposure or inhalation from air and dust. Such pathways could lead to different ratios of BPA-sulfate and BPA-glucuronide in serum, but the pharmacokinetics of these non-dietary exposure routes are poorly understood.

2.3.4 Higher concentrations of BPA metabolites in cord serum compared to maternal serum.

Although no statistical difference was observed between maternal and cord BPA-sulfate concentrations, BPA-glucuronide and total BPA metabolite concentrations (BPA-sulfate + BPA-glucuronide) were significantly higher in cord sera ($p<0.05$) compared to maternal sera using the *Wilcoxon Signed Rank* test which accounts for non-detect data (Figure 2-3). In general, 56% of cord serum had higher BPA metabolites than paired maternal serum, 37% of cord serum had lower BPA metabolites than paired maternal serum, and 7% of paired maternal and cord serum were both non-detect for BPA metabolites. The higher concentration of BPA metabolites in fetal serum lends further feasibility to the possibility that the fetoplacental compartment influenced the maternal serum metabolite profiles measured here.

Significantly higher conjugated BPA was also observed in fetal serum compared to maternal serum after continuous subcutaneous BPA exposure to pregnant rhesus monkeys.[31] In that study, clearance of conjugated BPA was slower from the fetus relative to maternal serum after oral exposure.[31] The slow clearance of BPA-glucuronide in fetal sheep (half-life: 28±6.4 hrs) compared to adults (half-life: 3.2±1.8 hrs) after infusion of BPA-glucuronide was also observed by Gauderat et al.[32] Furthermore, their results suggested a conjugation-deconjugation cycling of BPA in fetal compartment, which led to a 43% increase in fetal exposure to free BPA and a low but sustained free BPA exposure in the fetus with a highly prolonged hydrolysis-dependent plasma terminal half-life (>100 hrs).[32] The low placental permeability of BPA-glucuronide has moreover been noted in a human placenta perfusion study.[57] In addition to elimination of BPA metabolites through the placenta back to maternal circulation, the fetus may also excrete conjugated BPA metabolites to amniotic fluid. BPA has been observed in human amniotic fluid,[58] and elevated BPA (comparable to or higher than maternal serum) was detected in amniotic fluid after intravenous infusion or subcutaneous exposure during pregnancy in animal studies.[31,59] Nevertheless, the fetus can be re-exposed to this excreted BPA, either by swallowing the amniotic fluid or through dermal absorption.[60] Overall, the pharmacokinetics of BPA in the human fetus are not well

understood but are likely much different from adults. This may explain the lack of any statistical association between maternal and cord serum concentrations (p=0.80 for BPA-sulfate, p=0.31 for BPA-glucuronide, p=0.23 for total BPA metabolites), but the 1 to 2 days delay between maternal and cord blood sampling may also have contributed.

2.3.5 Significance of results.

Background contamination of samples with free BPA has widely been cited as a reason for not using serum samples in routine BPA exposure assessment.[33,61–63] Nevertheless, serum is the matrix of greatest biological significance to the developing fetus, and for BPA it may be important to understand exposure to metabolites, not only the total BPA exposure. The current study demonstrates that the major BPA metabolites can be measured in maternal and cord serum, and the high relative abundance of BPA-sulfate is different than anticipated from oral exposure studies in non-pregnant populations. These first data for BPA-metabolites in paired maternal and cord sera support previous separate studies in cord blood[48] and maternal urine, [51] which also point to this special BPA metabolite pattern, but for the first time also demonstrate higher total BPA-metabolites in cord serum compared to paired maternal serum. The cross-contamination of fetal cord blood from maternal blood could not be completely excluded, but the results suggested that future studies of BPA metabolites in serum should provide complimentary information to urinary biomonitoring of total BPA.

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Table 2-1 Total and free BPA in paired maternal and cord serum.

	Maternal serum		Cord serum	
	Total BPA	Free BPA	Total BPA	Free BPA
Detection frequency	75%	38%	52%	8%
GM [a]	0.31	0.12	0.24	NC[b]
Mean [a]	0.48	0.20	0.56	NC[b]
Range	<LOD[c]-1.87	<LOD[c]-1.04	<LOD[c]-4.26	<LOD[c]-1.52

[a] Units of GM and mean are ng/mL.
[b] Not calculated because of the low detection frequency.
[c] 0.12 ng/mL

Table 2-2 Mass transition, limit of detection (LOD), limit of quantification and procedural blank concentration of target analytes.

Compounds	Molecular weight	Mass transition	LOD[a] (ng/mL)	Retention time (min)	Blank concentration[b]
BPA	228	227>212	0.12	4.41	0.08 ng/mL
$^{13}C_{12}$-BPA-glucuronide	416	415>113	-	3.43	-
BPA-glucuronide	404	403>113	0.01	3.43	-
BPA-sulfate	308	307>227	0.01	3.63	-
BPA-bissulfate	388	387>307	0.20		-
$^{13}C_{12}$-BPA	240	239>224	-	4.41	-
BPS	250	249>108	0.03	3.02	-
BPAF	336	335>265	0.01	4.91	0.005 ng/mL
BPB	242	241>212	0.05		-

[a] the LOD were the higher value of either the signal-to-noise ratio of three (*S/N*=3) or the average blank signal plus 3 times the standard deviation in chromatograms of sample extracts from blank cow serum.

[b] mean value of 10 blank samples.

Table 2-3 Recovery of target analytes spiked to serum and extracted by SPE.

Compounds	Mean Recovery and RSD (%)				
	low level[a]	med level[b]	high level[c]	intraday RSD[d] (n=5)	interday RSD[d] (n=10)
Detected without enzyme digestion					
free BPA	84%	89%	105%	4.1	8.9
BPA-glucuronide	106%	99%	99%	5.9	8.4
BPA-sulfate	72%	90%	87%	3.3	6.7
BPA-bissulfate	64%	78%	74%	6.8	7.3
free BPS	62%	63%	70%	6.0	10.1
free BPAF	71%	80%	76%	3.6	7.3
free BPB	79%	81%	89%	5.0	9.2
Detected with enzyme digestion					
total BPA	80%	79%	83%	3.0	6.1
total BPS	71%	72%	75%	5.8	7.1
total BPAF	73%	80%	80%	4.7	8.1
total BPB	82%	78%	83%	3.5	8.3

[a] 1 ng/mL for BPA, 0.5 ng/mL for other target analytes (n=5).
[b] 5 ng/mL for BPA, 2 ng/mL for other target analytes (n=5).
[c] 10 ng/mL for BPA, 5 ng/mL for other target analytes (n=5).
[d] over 2 months.

Table 2-4 Detection frequency, geometric Mean (GM) and arithmetic mean of BPA metabolites, and total BPS in maternal and cord serum (n=61)

	BPA-sulfate	BPA-glucuronide	Total BPS
Maternal serum (ng/mL)			
Detection frequency	66%	36%	7%
GM	0.06	0.02	NC[a]
Mean	0.22	0.05	NC[a]
Range	<LOD-1.70	<LOD-0.76	<LOD-0.07
Cord serum (ng/mL)			
Detection frequency	59%	61%	12%
GM	0.08	0.04	NC[a]
Mean	0.54	0.12	NC[a]
Range	<LOD-4.27	<LOD-1.21	<LOD-0.12

[a] NC=Not calculated due to low detection frequency

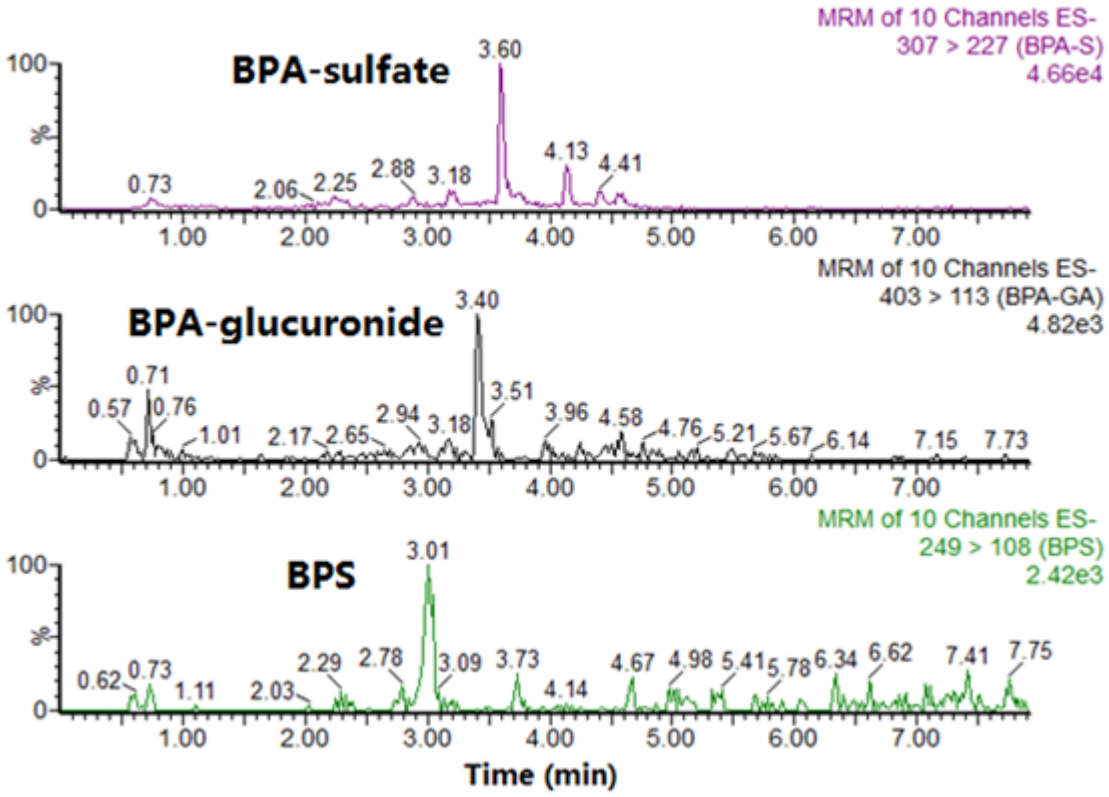

Figure 2-1 Example chromatograms of BPA-sulfate, BPA-glucuronide and BPS detected in maternal serum. Their concentrations were respectively 0.44 ng/mL (BPA-sulfate), 0.14 ng/mL (BPA-glucuronide) and 0.12 ng/mL (BPS).

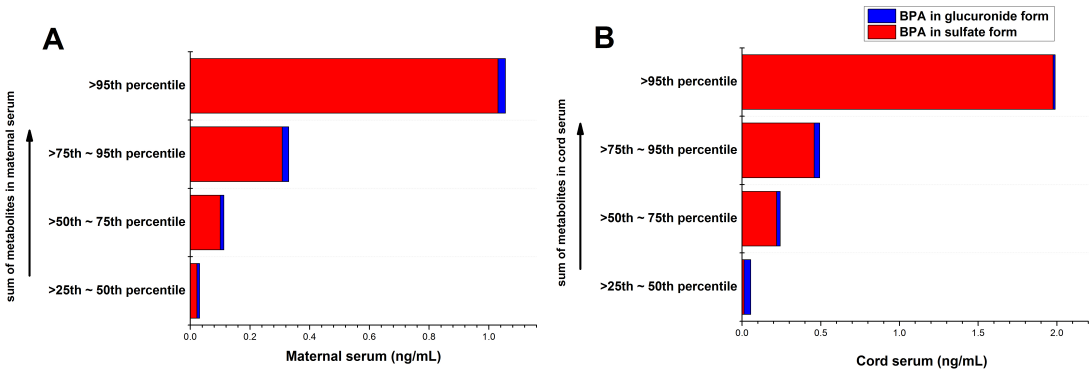

Figure 2-2 Cumulative geometric mean concentrations of BPA in sulfate and glucuronide forms in maternal serum (A) and cord serum (B) among various percentile ranks of sum of metabolites.

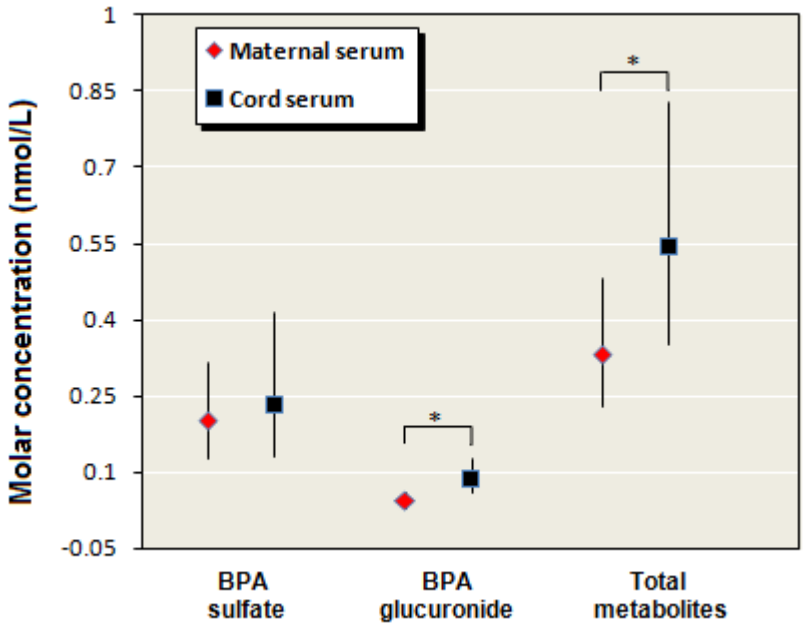

Figure 2-3 Geometric mean and 95% CI of BPA-sulfate, BPA-glucuronide and sum of BPA-sulfate and BPA-glucuronide (Total Metabolites) in paired maternal and cord serum. (* p<0.05)

3 Prolonged Exposure to Bisphenol A from Single Dermal Contact Events[⑥]

3.1 Introduction

BPA is a high production volume chemical [1] used for polycarbonate plastic manufacturing, or in epoxy resin linings of food and beverage containers, and as a developer in thermal paper, such as for shopping receipts and luggage tags at airports. BPA now occurs ubiquitously in the environment [2] and human populations are widely exposed to low levels.[3] According to national surveys in Canada (2012-2013), approximately 90% of the general population have detectable urinary concentration of BPA (>0.2 ng total BPA/mL).[4]

The toxicological hazard of BPA includes its activity as an endocrine disruptor.[5] In low-dose animal studies, effects have been shown on brain function, development and behaviour.[6,7] Epidemiology studies have shown associations between gestational BPA exposure and anxious, depressive and hyperactive behaviours in children,[8,9] but results are not consistent between studies.[10] Difficulties in these population-level studies include representative measures of exposure, and the within-person variability of urinary concentration of BPA.[11,12]

Diet has been regarded as the major human exposure pathway to BPA.[13,14] Toxicokinetic studies in humans show that after oral absorption, free BPA – regarded as the toxic form of BPA – is efficiently metabolized by first-pass metabolism to nontoxic metabolites which are quickly eliminated through urine.[15,16] The biological half-life of BPA following oral exposure is therefore less than 6 hrs.[15,16] However, Stahlhut et al. found that urinary BPA concentrations did not decline rapidly with fasting time in 1,469 participants from the 2003-2004 National Health and Nutrition Examination Survey (NHANES).[17] These findings suggested substantial non-dietary BPA exposure pathways in the general US population, and the authors moreover suggested that: (i) non-food sources need to be identified, and (ii) that risk assessments based on assumptions of a predominant oral exposure pathway may need reconsideration.[17]

Dermal exposure to BPA may be an important non-dietary exposure pathway in some people. High levels of BPA and related alternative chemicals (i.e. BPS) have been widely detected in paper products such as thermal paper receipts and paper currencies. [1,18–20] The latest exposure assessment from the European Food Safety Authority (EFSA) concluded that thermal paper was the second most important

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[⑥] A version of this Chapter has been published as *Environ. Sci. Technol.* **2017**, 51(17), 9940-9949.

source of BPA in all population groups above 3 years of age, contributing 7-15% of total average exposure.[21] Data from NHANES supports the link between occupational exposure from thermal paper and increased urinary BPA excretion.[22] Higher urinary BPA levels in cashiers was also observed previously,[23,24] and recently the dermal contact route was estimated to contribute 52-84% of total BPA exposure for cashiers.[25] Occupational exposure to BPA by the dermal route was also suspected as the cause of elevated (up to 2 orders of magnitude) urinary concentration of total BPA in thermal paper and paint product manufacturing.[26]

The pharmacokinetics of BPA in humans following dermal exposure are not clear. In vivo animal dermal absorption studies and in vitro skin penetration experiments have been used to extrapolate to humans, but with varying results. For example, one study concluded that dermal absorption of BPA was likely negligible as only 8.6% of the dose penetrated through the human skin after 24 hrs incubation,[27] while another study using a higher dose in human skin suggested that dermal BPA exposure may in fact be toxicologically relevant.[28] Similarly, one study with human skin samples concluded that absorbed BPA was not biotransformed (i.e. <3%),[28] while another study with viable human skin demonstrated 27% conjugation to glucuronide and sulfate.[29] As previously discussed, [27] the species, the dose and penetration times are important variables in these experiments. A current knowledge gap is therefore the lack of in vivo human data on dermal BPA absorption and subsequent rates of elimination following a realistic exposure scenario. Teeguarden et al. recently cited a dermal BPA exposure study using isotope labelled BPA in United States,[30] but to our knowledge this study is not yet publicly available.

In the current study, ethics approval was obtained to recruit six healthy male participants for controlled studies of dietary and dermal exposure using low doses of deuterated BPA (BPA-d_{16}). The participants handled simulated receipt paper containing isotope-labelled BPA at a concentration (25 mg BPA-d_{16}/g paper) similar to native receipt papers in various countries (i.e. 15-36 mg BPA/g paper).[18,20,23,24,31] After 1 week, participants returned to undergo a single dietary exposure (20 µg BPA-d_{16}), comparable to consuming 250 g canned soup [32] and approximately two orders of magnitude lower than the provisional TDI (25 µg/kg BW/day) established by the Food Directorate of Health Canada.[33] Blood and urine samples were collected before and after both exposures to compare and contrast the resulting pharmacokinetics.

3.2 Experimental section

I am responsible for the study design, participants recruitment, sample collection, sample analysis and data analysis.

3.2.1 Participant recruitment.

The study protocol was reviewed and approved by the Health Research Ethics Board of the University of Alberta (Study ID: Pro00051789). Only men were recruited to avoid any concerns for reproductive or developmental effects [8,9] in women of child-bearing age. In a pilot study, a single adult male (age 41) provided informed consent to manually handle simulated receipt papers (25 mg BPA-d_{16} /g paper) and native thermal receipts, followed by hand-wipes, to test paper-hand transfer of BPA-d_{16} from the simulated thermal receipts. For the subsequent full study, six healthy male participants were recruited (age 25 to 35, weight 55 to 94 kg) using poster advertisements at the University of Alberta. Three of the participants were Chinese, one was Arab, and two were Caucasian. After explaining the study purpose and procedures, signed informed consent forms were collected prior to exposures. All participants were asked to refrain from alcoholic beverages, medications, any canned food and touching thermal paper receipts for 24 hrs prior to arriving at the clinic and during the sample collection period. A questionnaire was completed documenting each participant's dietary information for 24 hrs prior to the dermal exposure, and the participants were asked to eat similar foods in the 24 hrs preceding the dietary oral exposure, one week later. Food and beverages consumed during the study were also recorded in detail by each participant.

3.2.2 Dermal exposure.

After collection of a control urine and serum sample from each participant, the non-dominant hand of each participant was carefully wiped with a water moistened Kimwipe to check for any background exposure. After 5 min to allow the hand to dry, the participants were asked to hold and handle a simulated thermal receipt (6×10 cm soft notebook paper containing 20 mg BPA-d_{16}) with their non-dominant hand for exactly 5 min. The start time of handling the simulated receipt was set as time 0. To avoid unintended oral exposure, the subjects were told not to eat or touch their mouths with the contaminated hand or paper during the handling. After the simulated receipts had been recollected and placed in plastic bags, the participants all put a single nitrile glove on the exposed hand for 2 hrs. This was to prevent any incidental hand-to-mouth exposure, and furthermore to prevent contamination of urine samples during collection. The participants remained seated in a conference room for the following 2 hrs, but were free to use the washroom.

After 2 hrs, the nitrile glove was removed and the contaminated hand was carefully wiped again with a water moistened Kimwipe to evaluate transfer of BPA-d_{16} to the hand. All participants were then instructed to immediately and carefully wash their hands with soap in the washroom. The Kimwipes (one collected before, and one after the administration) were extracted in methanol and analyzed for BPA-d_{16}.

One month after the above dermal administrations, one participant (P1) agreed to return for a repetition of the dermal exposure study exactly as above, except with a longer sampling period for urine and serum, as described below in 'Urine and blood collection'.

3.2.3 Dietary exposure.

One week after the above dermal administration, 5 of 6 participants returned to participate in the dietary exposure. After providing a control urine sample, each participant then ingested a cookie containing 20 µg BPA-d_{16}. This dietary dose (0.22-0.36 µg/kg BW/day) is higher than the average estimated daily intake (EDI) of BPA from food (0.055 µg/kg BW/day) for the general population in Canada,[34] but it is approximately two orders of magnitude lower than the provisional TDI of 25 µg/kg BW/day established by the Food Directorate of Health Canada,[33] and approximately 10 times lower than the latest TDI (4 µg/kg BW/day) of the EFSA.[35]

3.2.4 Urine and blood collection.

In addition to providing a pre-exposure urine sample, participants collected post-exposure urine samples for up to 2 days after exposure in both dermal and oral studies. Participants were given a written standard operating procedure and were asked to collect the total volume of each urine event in 400 mL disposable plastic beakers (Sigma-Aldrich, St. Louis, MO). After recording time of collection and total urinary volume, approximately 30 mL of the total urine was poured into a 60 mL labelled collection cup; the rest of the urine was discarded. Participants were asked to return all urine collection cups to the lab within 48 hrs. In the lab, 5 mL subsamples of each urine sample were transferred to cryovials by plastic pipette and stored at -80^{o}C until analysis. To estimate urinary dilution, a 1 mL aliquot of each urine sample was submitted to Laboratory Services at University of Alberta Hospital for creatinine analysis (Synchron LX® Systems, Beckman Coulter).

In addition to providing a pre-exposure serum sample, three post-exposure blood samples were collected from each participant over a 7.5 hrs period in the clinic following dermal and oral exposure. In the dermal exposure study where exposure was only to one-hand, blood was drawn from the exposure

arm of participants P1, P4, and P5, and from the non-exposure arm of participants P2, P3 and P6. Blood was collected into red cap BD Vacutainer serum tubes. After clotting, collection tubes were centrifuged and 2.0-3.0 mL serum was transferred into two 1.5 mL microcentrifuge tubes for each blood sample and stored at -80^{o}C until analysis.

In the follow-up dermal exposure study with one participant (P1), a pre-exposure urine sample was collected in addition to all urine samples for 5 days post-exposure. First morning urinary voids were also sampled for another 4 days (9 days total urinary monitoring). Furthermore, in the same individual, two blood samples were collected from the exposure arm at 22 hrs and 51 hrs post-exposure. Samples were processed and stored as above.

3.2.5 Materials and methods

3.2.5.1 Chemicals and reagents.

BPA-d_{16} (98%), bisphenol A mono-β-D-glucuronide (BPA-glucuronide; 95%), and β-glucuronidase enzyme from *Helix pomatia* (≥100,000 units/mL β-glucuronidase; ≤7500 units/mL sulfatase) were obtained from Sigma-Aldrich (St. Louis, MO). Bisphenol-A-(diphenyl-$^{13}C_{12}$) ($^{13}C_{12}$-BPA, 99%) was purchased from Cambridge Isotope Laboratories (Andover, MA). Alcohol (56% ethanol, Chinese white spirit, Beijing, China) was purchase from a local liquor store in Edmonton, Canada. High performance liquid chromatography (HPLC) grade water and methanol were acquired from Fisher Scientific (Fair Lawn, NJ). HPLC grade ammonium acetate and analytical grade formic acid (98%) were obtained from Sigma-Aldrich (St. Louis, MO). Denatured (95%) ethyl alcohol and ammonium hydroxide (NH_4OH, 25% in water) were purchased from Fisher Scientific (Fair Lawn, NJ).

3.2.5.2 Preparation of cookies and of simulated receipts.

To avoid major interference from background environmental BPA exposure, and to allow us to conduct this study at low doses, BPA-d_{16} was used in the exposures. For dietary exposures, solid BPA-d_{16} standard was dissolved in Chinese white spirit to prepare a 1 mg/mL stock solution, and 20 µL of this (20 µg BPA-d_{16}) was spiked onto single cookies. For dermal exposure, solid BPA-d_{16} standard was dissolved in 95% denatured ethyl alcohol to make a 20 mg/mL stock solution, and 1mL of this (20 mg BPA-d_{16}) was manually spread dropwise on 6×10 cm soft notebook papers to achieve the approximate concentration of 25 mg BPA-d_{16}/g paper. The dosage on the prepared paper (25 mg/g paper) was comparable with the maximum BPA concentrations on thermal receipts in the Unites States (max: 36 mg/g), Switzerland (max:

17 mg/g), France (max: 18 mg/g) and China (max: 15 mg/g).[18,20,23,31] The spiked cookies and simulated receipt papers were left to air dry overnight prior to the exposures.

3.2.6 Sample preparation.

Two 500 µL aliquots from each urine sample were used for analysis of free and total BPA-d_{16} (conjugated plus free species) separately. For analysis of total BPA-d_{16}, one 500 µL aliquot of sample was mixed with 5 µL of 0.5 ng $^{13}C_{12}$-BPA and 200 µL of 1 M ammonium acetate buffer containing 2 µL β-glucuronidase enzyme, which was enough to digest 100 ng of the respective glucuronide, and 5 ng of the respective sulfate. The mixture was incubated at 37 °C overnight to deconjugate the metabolites, and 300 µL of 1 M formic acid was added to bring the final volume of each sample to 1 mL. For analysis of free BPA-d_{16}, the other aliquot was mixed with 5 µL of 0.5 ng $^{13}C_{12}$-BPA, 300 µL of 1 M formic acid and 200 µL of water. $^{13}C_{12}$-BPA was used as internal standard for quantification of total and free BPA-d_{16}. For serum samples, enzymatic deconjugation was used as for urine samples, followed by offline solid phase extraction (SPE). The SPE method was described previously.[36]

3.2.7 Online SPE/HPLC/Orbitrap analysis.

Quantitative analysis of BPA-d_{16} was achieved using online SPE coupled to HPLC and an Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA) operating with an atmospheric pressure chemical ionization source in negative ionization mode. The mobile phases in online SPE and HPLC were water and methanol. Injection volume was 300 µL of sample, containing 150 µL of either urine or serum). The gradient used in online SPE-LC was described in one of our former article.[37] Recovery of BPA-d_{16} was in the range of 88% to 108% with relative standard deviation between 3% and 13% (Table 3-1). The regression coefficients of the standard curves of BPA-d_{16} were always > 0.99 for urine and serum.

3.2.8 Data analysis.

Data analysis was performed with Stata 12.0, and the level of significance for all of statistical tests was p<0.05. Non-detect results were substituted by ½ the LOD for data analysis and all non-detects are colour coded in all Figures. In statistical tests, the percentage of data below LOD was always less than 15%. The Wilcoxon-Mann-Whitney test was used to compare sampling times of maximum urinary total BPA-d_{16} concentration (T_{max}) between dermal and dietary exposure studies for P1 to P4 who had detectable urinary total BPA-d_{16} in both dietary and dermal exposure studies. For comparison of cumulative excretion, in the dermal exposure study linear regression was used to calculate the slopes of

individual (P1-P4) cumulative excretion curves using all urinary total BPA-d_{16} concentration data from P1 to P4. In the dietary exposure, slopes of the individual (P1-P4) cumulative excretion curves were calculated from the pre-exposure urine and the first three urine samples collected after the exposure time (i.e. samples collected before the plateau). The Wilcoxon-Mann-Whitney test was then used to compare slopes between dermal and dietary exposure studies. I appreciate the kind assistance from Dr. Irina Dinu and Elham K. Moez (School of Public Health, University of Alberta) in statistical analysis of the data.

3.3 Results

3.3.1 Transfer of BPA-d_{16} to hand from simulated receipt paper.

To confirm exposure to the skin, BPA-d_{16} was extracted and analyzed in pre- and post-exposure hand wipes (Table 3-2). Due to the nature of the hand-wipe sample, these data are not highly quantitative, but are useful to evaluate inter-individual variability in extent of test chemical transfer from simulated receipt paper to skin.

In a pilot study with one male, the dermal exposure condition (paper containing 25 mg BPA-d_{16}/g handled for 5 min, glove for 2 hrs) resulted in 0.24 µg BPA-d_{16} in a hand wipe, similar to the amount of BPS (0.61 µg) transferred from a native receipt paper (containing 17 mg BPS/g paper) handled in the same manner. Thus the simulated receipt papers were deemed to be realistic for the full study.

In the full study with 6 individuals, there was large inter-individual variability in BPA-d_{16} mass collected on hand wipes (range 0.02 - 3.0 µg). This may be due to the relative vigour with which each participant handled the receipt paper, moisture content of the hand ,[18] or variation in lipid and keratin content of the stratum corneum. Biedermann et al. reported that 0.2 to 6 µg of BPA was transferred to two fingers after holding a thermal receipt for 5 seconds.[18] Different from our study, they tested the BPA level on two fingers immediately after the contact, while I wiped the hand 2 hrs after the exposure and did not test for residual BPA-d_{16} in the gloves. Thus, the BPA-d_{16} mass on hand wipes was likely only a fraction of the BPA-d_{16} transferred from simulated receipt papers to the hand.

3.3.2 Urinary excretion of total BPA-d_{16} following dermal exposure.

No traces of BPA-d_{16} were detectable in control hand wipes, in gloves or in any sample collection or storage materials. Procedural blanks (HPLC grade water spiked with internal standards) were analyzed with every batch of up to 20 samples and no BPA-d_{16} was detectable in any of the blanks. Pure water

was injected after high spiked urine samples (100 ng/mL BPA-d_{16}) and no instrumental background or carryover was ever observed. Food consumption was recorded during the dermal exposure study period, and the variance in urinary BPA-d_{16} concentration was not explained by recorded meal times (Figure 3-1), confirming that hand-to-mouth exposure of BPA-d_{16} was insignificant.

Total BPA-d_{16} was not detectable in any participant's pre-exposure control urine sample. Total BPA-d_{16} was also not detectable in any post-exposure urine samples from participant P6, whose hand-wipe had the lowest mass of BPA-d_{16} recovered from any participant (Table 3-2). Thus, data from P6 in the dermal exposure study were not included in any analysis. For the other 5 participants, maximum concentrations (C_{max}) within the initial 2 days of urinary monitoring occurred between 15 and 34 hrs and ranged from 0.18 to 4.6 ng/mL (Table 3-3). These urine concentrations were comparable to the GM of urinary total BPA (i.e. 1.1 ng/mL) in the general Canadian population.[4] Overall, the excreted total BPA-d_{16} in urine following dermal exposure showed a slow increase to the C_{max}, followed by a slow decrease (Figure 3-2). In fact, one week after the dermal exposure, the 'control' urine samples collected prior to the dietary exposure revealed that BPA-d_{16} was still detectable in the urine samples from 3 of 5 remaining participants, in the range of 0.11 to 0.37 ng/mL urine (Figure 3-3).

Despite the variability in BPA-d_{16} urinary concentrations between participants, the cumulative excretion of BPA-d_{16} from all 5 participants with detectable levels in urine showed an increasing trend over 48 hrs, beginning between 5 and 10 hrs post-exposure (Figure 3-4). Large inter-individual variation in human skin penetration efficiency of BPA has been noted previously,[28] and to some extent this may explain the high variability observed here between participants. However, the variability in urinary concentration between the participants may also relate to the mass of BPA-d_{16} transferred to the hand, which I evaluated semi-quantitatively in hand wipes after 2 hrs. For example, the highest BPA-d_{16} level on the hand wipes was for participant P1, who also had the highest urinary C_{max}.

The unexpected findings of prolonged BPA exposure following a single dermal exposure led to a follow up study, albeit with only one out of the 6 participants agreeing to repeat the dermal exposure and to be monitored for 9 days (212 hrs). This participant (P1) repeated the dermal exposure one month after the previous dermal administration, and urinary BPA-d_{16} was not detectable in his control urine collected prior to the repeated exposure. Total BPA-d_{16} first became detectable in urine at 4.6 hrs after the dermal exposure and increased to 4.3 ng/mL by 21 hrs, showing very similar concentrations and cumulative excretion (in Figure 3-4) compared to this participant's first trial. Continued sampling showed that urinary BPA-d_{16} concentration remained elevated and was detectable in every sample over

the next 5 days (Figure 3-1). Cumulative excretion of BPA-d_{16} in this time period of 116 hrs was close to linear (Figure 3-4), suggesting slow penetration of BPA-d_{16} through the skin, and with the skin passively dosing the circulating blood. The cumulative excreted BPA-d_{16} in 116 hrs was 12 μg, indicating that at least 12 μg BPA-d_{16} penetrated into the skin after 2 hrs before hand-wipes and washing with soap.

Continued monitoring of the single participant showed an apparent diurnal urinary concentration pattern over the first 5 days, whereby concentrations were relatively higher in the first morning's urine and decreased at night (Figure 3-1). For the following 4 days, only the first morning's urinary void was collected. BPA-d_{16} was detectable in all these morning voids with concentrations ranging from 1.2 μg/g creatinine (5th morning) to 0.11 μg/g creatinine in the final sample collected. A trendline through the first morning urinary data clearly shows a decreasing trend of excreted total BPA-d_{16} from day 3 to day 9 (Figure 3-1). Urinary data from this follow up dermal exposure study are compared to the national survey of urinary concentration of BPA in Canada (2012-2013) [4] in Figure 3-5.

BPA is rapidly and almost completely excreted in urine within 24 hrs after oral exposure in human or subcutaneous administration in rhesus monkey,[15,16,38] and excreted urinary total BPA is therefore the biomarker of choice to estimate daily BPA intake.[39] From the slope of the cumulative urinary excretion curves following dermal exposure, the mass of BPA-d_{16} excreted per day was in the range of 0.10 to 3.0 μg for the 5 participants that had detectable BPA in urine. Thus, the estimated daily intake (EDI) of BPA-d_{16} in the present dermal exposure study was in the range of 0.002 to 0.046 μg/kg BW/day for the 5 participants. These numbers approach the average dietary EDI for the general Canadian population (0.055 μg/kg BW/day) [34] and the recently established maximum allowable dose level (MADL) in California of 3 μg/day for dermal exposure to BPA from solid materials.[40]

3.3.3 Urinary excretion of total BPA-d_{16} following dietary exposure.

Out of the 6 participants who participated in the dermal exposure study, 5 returned one week later to participate in the dietary exposure study. As discussed above, due to the unanticipated prolonged exposure following the dermal exposure, BPA-d_{16} was still detectable at low levels (0.11 - 0.37 ng/mL) in 3 of 5 control urine samples collected prior to dietary exposure. These low levels, however, did not interfere with the analysis of the dietary exposure study due to the higher ingested dose.

Urinary total BPA-d_{16} reached C_{max} (11 - 76 ng/mL) within 1.0 to 5.5 hrs for each participant. Compared to the dermal exposure pharmacokinetics, the excreted total BPA-d_{16} in urine showed a rapid increase to a maximum 1.0 – 4.0 hrs post-exposure, followed by a rapid decline of total BPA-d_{16} (Figure 3-2). Approximately 50% of the dietary dose was excreted within 1 to 3 hrs, and on average 102% (range

86 - 113%) of the dietary dose was recovered in urine within 24 hrs (Figure 3-6). Self-reported total urinary volumes from P6 were too low (330-400 mL urine/day) to be accurate, particularly compared to other participants, thus I could not reliably calculate cumulative excretion of total BPA-d_{16} for participant P6.

3.3.4 Free BPA-d_{16} in urine following dermal and dietary exposures.

Free BPA-d_{16} was analyzed in urine following the dermal and dietary exposures. In urine samples with detectable total BPA-d_{16} following dermal exposure, free BPA-d_{16} was detectable at 9% detection frequency in the first study, and in 26% of samples in the follow up study with one person. Overall, the range of free BPA-d_{16} in urine after dermal exposure was 0.02 to 0.52 ng BPA-d_{16}/mL. By comparison, in urine samples that contained detectable total BPA after the dietary exposure study, free BPA-d_{16} was detectable at 26% detection frequency, ranging from 0.02 to 0.88 ng BPA-d_{16}/mL. In both studies, urine samples corresponding to C_{max} of total BPA-d_{16} also had the maximum concentrations of free BPA-d_{16} (Figure 3-7). As shown in Figure 3-8, the proportion of detected free BPA-d_{16} in urine as a proportion of total BPA-d_{16} following the dermal exposure was higher than in the dietary exposure. Because free BPA-d_{16} was only detected in urine of three participants after the dermal exposure, a statistical test was not applied to these data and they are presented visually by participant (Figure 3-8).

3.3.5 Free and total BPA-d_{16} in serum following dermal and dietary exposures.

Total and free BPA-d_{16} were also analyzed in serum collected following the dermal and dietary exposures. Following dietary exposure, C_{max} of total BPA-d_{16} in serum (0.02 - 0.09 ng/mL) reached C_{max} rapidly (<4 hrs) and the serum total BPA-d_{16} decreased to below LOD after 7.5 hrs.

Importantly, no free BPA-d_{16} was ever detected in serum following dietary exposure (Table 3-4). This result is consistent with what has been predicted from pharmacokinetics of other studies [15,16] that, given current dietary exposures, free BPA should be below 0.001 ng/mL, which is much lower than our LOD (i.e. 0.015 ng/mL). Based on the LOD for the current analytical method, the percentage of free BPA-d_{16} in serum during the dietary exposure can therefore only be estimated as <<18%.

In the dermal exposure study with all participants, where blood was only collected for 7.5 hrs post-exposure, no total or free BPA-d_{16} was ever detectable in serum. However, in the repeated dermal exposure study with one participant, serum was sampled later, at 22 and 51 hrs post-exposure. In these two samples the % free BPA-d_{16} was greater than 50% (Table 3-4), and no traces were detected in blank serum (Figure 3-9). The higher proportion of free BPA-d_{16} in serum for this participant following dermal

exposure is consistent with the urinary free BPA-d_{16} data in urine, whereby the free BPA-d_{16} proportion in urine following dermal exposure was generally higher than in the dietary exposure (Figure 3-8). In a previous report, holding thermal receipt papers after using hand sanitizer resulted in significant free BPA in serum.[41]

3.4 Discussion

This is the first report of an isotope-labeled BPA dermal exposure study in human participants. The use of the isotope-labeled BPA provided lower LOD (i.e. no signal in blanks) and lends additional confidence that the results are not biased by background BPA exposures. Moreover, use of the isotope labeled BPA allowed us to design the study with relatively low doses that are similar to those encountered by average people. The concentration of BPA-d_{16} on simulated receipt papers in this study is similar to native receipt papers analyzed in other studies.[18,20,23,24,31] Moreover, in this study the hands were carefully wiped after 2 hrs exposure, and the participants were instructed to wash their hands immediately after the hand wiping. I did not test for residual BPA-d_{16} on the hands after washing with soap, but Fan et al. found that more than 60% of BPA could be removed by rubbing hands in water for 10 s, and that more than 80% BPA could be cleaned by rinsing with water and lotion.[19]

National biomonitoring of Canadians (2012-2013) revealed a GM of 1.1 ng/mL urinary concentration of total BPA (95% CI: 1.0 – 1.2 ng/mL; 95th percentile at 6.6 ng/mL).[4] In the current study of dermal exposure, urinary C_{max} of BPA-d_{16} over the first 48 hrs (n=5) was 0.18-4.6 ng/mL, thus showing some overlap of the current study with known urinary concentrations in Canadians. Nevertheless, the GM of urinary concentration of BPA-d_{16} over 48 hrs following dermal exposure was 0.12 ng/mL (95% CI: 0.07-0.19 ng/mL), which is lower than urinary concentration of BPA in general Canadians.[4] In the real world, people handling a receipt every few days, or those who handle receipts and do not wash their hands for a longer time, may achieve higher total BPA in urine than demonstrated in the current study.

Due to various limitations and uncertainties in the dermal exposure conditions, the concentrations of BPA in urine are less important results than the observed pharmacokinetics. For example, although the concentration of BPA on the simulated thermal receipts was realistic, the handling time (5 min) and time before handwashing (2 hrs) were arbitrary. Longer handling times and longer times before hand washing would likely lead to more BPA transfer and absorption, respectively, but the importance of these factors was not studied. Moreover, the effect of the nitrile glove on the extent of BPA absorption was not studied. It is possible that the wearing of the glove for 2 hrs led to higher humidity on the skin and may

therefore have altered BPA absorption. While all these factors may have biased the resulting urinary concentrations high or low, these should not have had any impact on the subsequent pharmacokinetics after the glove was removed and hands were washed at 2 hrs.

Large differences in pharmacokinetics were observed between the dermal and dietary exposure pathways. From the dietary pathway, total BPA-d_{16} reached peak concentrations in serum within 4 hrs (Table 3-4) and >96% of administrated BPA-d_{16} was excreted in urine within 12 hrs (Figure 3-6). By contrast, total BPA-d_{16} was not even detectable in serum within 7.5 hrs post dermal exposure, but was indeed detectable by 22 hrs and was still present after 51 hrs at a comparable concentration (0.29 and 0.30 ng/mL total BPA-d_{16}) (Table 3-4). Among the 4 participants with data from both dermal and dietary exposures, I could detect a statistically significant difference in the pharmacokinetics based on the T_{max} (Figure 3-3) and slope of the cumulative excretion curves (Figure 3-4) between the two routes of exposure (p<0.05); despite the small number of participants, both tests had high power (>0.95, based on 5% type I error). Curves of excreted total BPA-d_{16} in urine after dietary and dermal exposure (Figure 3-2) clearly demonstrated the prolonged exposure resulting from the slow dermal absorption.

The slow absorption of BPA from dermal exposure was also observed in vitro. Specifically, Demierre et al. reported only 8.6% of BPA penetrated through the human skin after 24 hrs incubation.[27] The absorbed dose of total BPA was 0.7% after 10 hrs incubation in pig skin [42] and 13% after 48 hrs in human skin.[43] A relatively higher absorption of BPA (46% of the total dose) was reported by Zalko et al. in viable human skin explants with longer incubation times (72 hrs).[29] It is important to note that the absorbed dose in these vitro studies was calculated from recoveries of BPA which penetrated through the skin into receptor fluids, and do not include the proportion of residual BPA accumulated inside the skin. For example, more than 30% of the dose was recovered in stratum corneum after 24 hrs incubation in human skin.[27] Furthermore, a rat percutaneous absorption study showed that residual BPA in skin at the end of a dermal exposure remained available for resorption into circulation for at least 64 hrs, and thus that the skin acted as a reservoir for BPA to extend the apparent biological half-life.[28] This is consistent with the current study whereby cumulative excretion of BPA-d_{16} was close to linear over 2-5 days post dermal exposure, even after hand washing.

Free BPA is generally regarded as the most toxic form of BPA [44] and was reported to comprise less than 1% of total BPA in blood following oral exposure.[15,16] As a result, arguments have been made that the adverse effects observed in animal models following subcutaneous dosage are questionable, because efficient first-pass metabolism following an oral exposure in humans would effectively decrease

or eliminate free BPA in systemic circulation.[45–47] Although data are limited in our study (2 samples from the same person), a high percentage of free BPA-d_{16} (greater than 50%) was observed in the two sera collected at 22 and 51 hrs post-dermal exposure (Table 3-4), suggesting a lower biotransformation efficiency of BPA following dermal exposures. Low biotransformation efficiency of BPA was also observed by Marquet et al. in an vitro study, in which unconjugated BPA accounted for >97% of the total BPA in the receptor fluid after 24 hrs exposure in human skin.[28] Relatively higher metabolism efficiency (i.e. 27% of the dose) was observed by Zalko et al. in viable human skin explants after 72 hrs of incubation,[29] but even this data is still far lower than following oral exposure. [15,16] Thus, dermal or subcutaneous BPA exposure in animal studies may not necessarily be an unrealistic model for consideration in risk assessments.

The highest daily intake following dermal contact conditions of the present study was 0.046 μg/kg BW/day (from P1), which was comparable to the average EDI (0.055 μg/kg BW/day) from diet for the general population in Canada [34] and equal to the MADL for dermal exposure of BPA from solid materials (3 μg/day, equal to 0.046 μg/kg BW/day for P1) established by OEHHA in California.[40] Based on previous limited data on the occurrence, migration and transfer of BPA from thermal paper, the EFSA estimated average exposure from dermal contact of thermal paper for adults to be 0.018 μg/kg BW/day, and high exposure to be 0.16 μg/kg BW/day.[21] This is consistent with the daily intakes in the present study following a single dermal contact event. Nevertheless, the prolonged exposure from single dermal contact events, as shown in this study, means that in the real world that exposures could be cumulative, and higher steady state exposure from repeated dermal contact events must also be considered.

Biomonitoring-based approaches were also used to evaluate BPA exposure from thermal paper in several studies. Increased exposure to BPA was found in workers who had occupational exposure to BPA through inhalation and dermal pathways.[26,48] For example, Heinälä et al. reported high concentrations of urinary total BPA in post-shift and next-morning samples from workers in a thermal paper factory, despite low airborne concentrations of BPA, suggestive of a dermal exposure pathway.[26] Data from NHANES 2003-2004 survey showed a positive correlation between occupational exposure to thermal paper and urinary concentration of BPA in females.[22] A significant increase in urinary concentration of total BPA after handling thermal paper receipts was also observed by Ndaw et al.[24] and Ehrlich et al.[49] Porras et al. evaluated BPA exposure from thermal receipts by analyzing the increase in native BPA concentrations in urine after handling thermal receipts, and concluded that the maximum EDI of BPA after handling thermal paper was less than 0.2 μg/kg BW/day.[50] However, the background level of BPA

in their reference group was high, in the range of 0.02-0.10 µg/kg BW/day, which may obscure the true increased EDI in their dermal exposure group. Thayer et al. reported that urinary concentration of BPA from cashiers was often lower in post-shift samples than in "pre-shift" (off-duty) samples, but that post-shift urinary BPA was significantly higher than in non-cashiers.[23] Given results of the current study, the "post-shift" samples (collected within 2 hrs of completing a work shift), might be too early to catch the maximum urinary concentrations, and the "pre-shift" samples (collected at least 24 hrs following a shift), were likely biased from BPA exposure in their previous work shifts. Thus, the results of the present study may be useful in design and interpretation of future BPA exposure studies of cashiers.

Human biomonitoring of BPA exposure has generally relied on detection of total BPA in urine. However, based on the present results, total BPA in urine may not be a good estimate of total or free BPA in systemic circulation resulting from a mixture of oral and dermal exposure. The serum concentrations of free BPA could be important to track, and if done carefully (without contamination) could inform about the pathway of exposure. Avoiding contamination of free BPA during sampling of blood is difficult to achieve,[51,52] and it must be noted that total and free BPA-d_{16} in serum following the present dermal exposure study was very low (0.02 – 0.03 ng/mL), and would not be detectable by today's methods with LODs in the range of 0.05-0.35 ng/mL.[30,53] Improved method sensitivity with continued careful control of background BPA contamination are required for biomonitoring BPA in blood of the general population.

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Table 3-1 Mass transition, limit of detection (LOD) and recoveries of target analytes.

Compounds	Mass transition	urine			serum		
		LOD (ng/mL)	Recovery (0.1ng/mL)	Recovery (2ng/mL)	LOD (ng/mL)	Recovery (0.5ng/mL)	Recovery (1ng/mL)
BPA-d_{16}	241>222.1459	0.015	93%	104%	0.015	108%	88%
$^{13}C_{12}$-BPA	239>223.1168	-	-	-	-	-	-

Table 3-2 Mass of BPA-d_{16} (μg) recovered on hand wipes before and after dermal exposure for the 6 male participants. P1-I and P1-II are the results of first and second dermal exposure on one participant.

Participant ID	Before dermal exposure (μg)	After dermal exposure (μg)
P1-I	ND[a]	0.87
P1-II	ND[a]	3.0
P2	ND[a]	0.34
P3	ND[a]	0.17
P4	ND[a]	0.17
P5	ND[a]	0.07
P6	ND[a]	0.02

[a] Not detectable

Table 3-3 Urinary concentrations and pharmacokinetic parameters for male participants who completed dermal and dietary exposures to BPA-d_{16}.

	Dermal exposure[a] (n=5)	Follow up dermal exposure[b] (n=1)	Dietary exposure (n=5)
Pre-exposure total BPA-*d_{16}* (ng/mL)	<LOD	<LOD	<LOD - 0.37
Post-exposure total BPA-*d_{16}* C_{max} (ng/mL)	0.18 - 4.6	6.3	11 - 76
Post-exposure total BPA-*d_{16}* T_{max} (hrs)	15 - 34	69	1.0-5.5
Post-exposure free BPA-*d_{16}* C_{max} (ng/mL)	<LOD-0.21	0.52	<LOD-0.88
Detection frequency of total BPA-*d_{16}*	78%	98%	94%
Detection frequency of free BPA-*d_{16}*[c]	9%	26%	26%
% as free BPA-*d_{16}* (mean ± SD)[d]	2.3% ± 1.6%	2.8% ± 2.1%	0.75% ± 0.39%

[a] urine monitoring over 2 days in 5 participants. Data for the 6th participant were excluded from this table due to non-detects in every sample.

[b] urine monitoring over 9 days in 1 participant (all urine events first 5 days, first morning void next 4 days).

[C] it is the detection frequency of free BPA-*d_{16}* in urine samples with detectable total BPA-*d_{16}*.

[d] calculated post-exposure in urine samples with detectable free BPA-*d_{16}* as proportion of total BPA-*d_{16}*.

Table 3-4 Serum concentrations and pharmacokinetic parameters for male participants who completed dermal and dietary exposures to BPA-d_{16}.

	Total BPA-d_{16}	Free BPA-d_{16}	% (Free BPA-d_{16} / Total BPA-d_{16})
Dermal exposure			
Concentration over 7.5 hrs (n=5, ng/mL)	< LOD	< LOD	-
Concentration at 22 hrs (n=1, ng/mL)	0.029	0.018	62%
Concentration at 51 hrs (n=1, ng/mL)	0.030	0.025	84%
Oral exposure			
Detection frequency (n=5)	67%	-	-
T_{max} (h) over 7.5 hrs (n=5)	1.2 -3.9	< LOD	-
C_{max} (ng/mL) over 7.5 hrs (n=5)	0.016 - 0.085	< LOD	<18% [a]

[a] Calculated as upper limit by LOD/C_{max}.

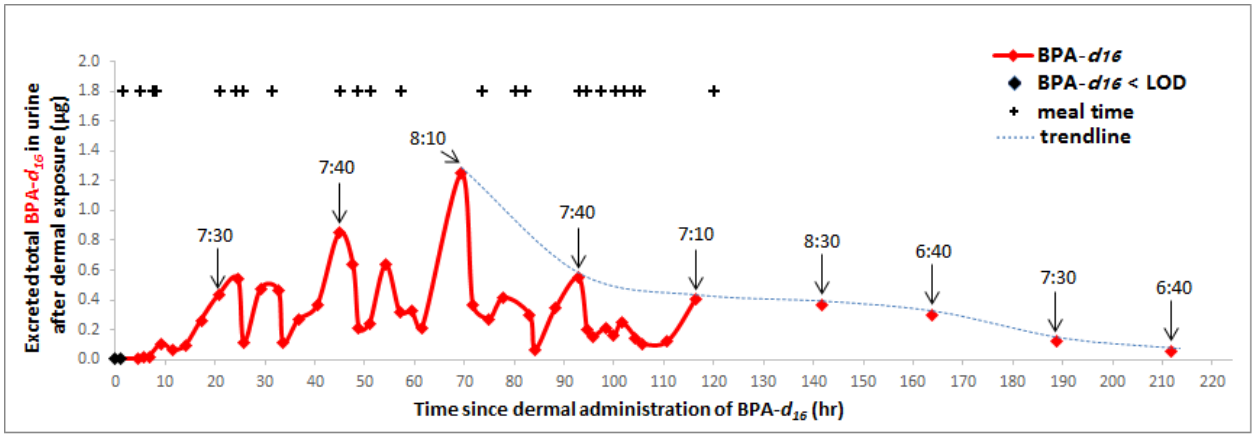

Figure 3-1 Excreted total BPA-d_{16} in urine after dermal exposure (µg) for one participant over 9 days after handling a simulated receipt paper (25 mg BPA-d_{16} /g paper). All urine events were collected in the first 5 days (116 hr) and first morning urine samples were then collected to day 9. First morning urine events are indicated by arrows and sampling time. The trendline of the decrease in first morning urine concentration is shown in blue dot line. Recorded meal times over 5 days are also indicated.

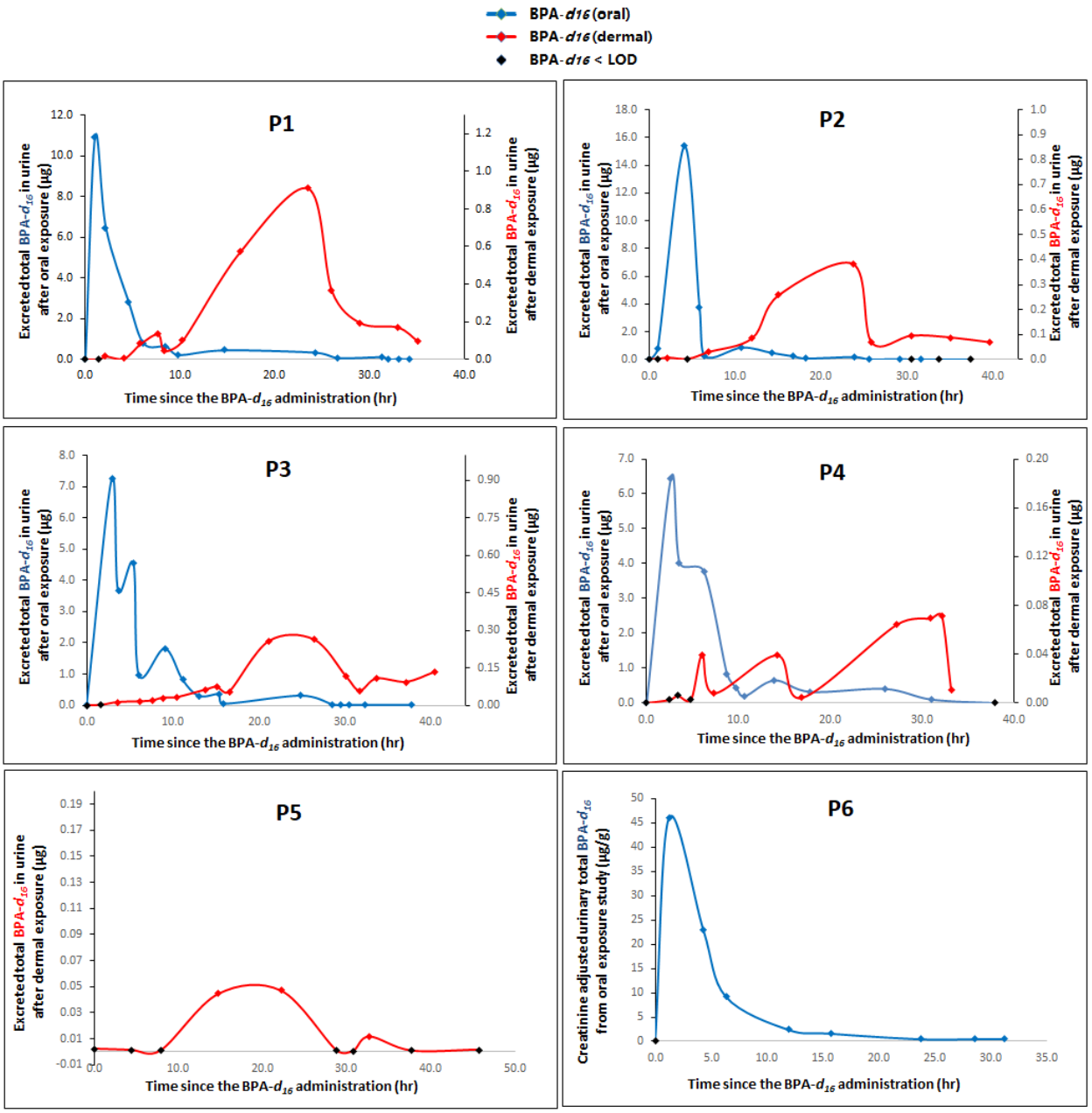

Figure 3-2 Excreted total BPA-d_{16} in urine samples (µg) collected within 41 hrs following a single dietary exposure (blue) and single dermal contact event (red). Non-detects are colour coded in black. As P5 did not join the dietary exposure study, I only plot his results of dermal exposure. P6 joined both dermal and dietary exposure studies, but BPA-d_{16} was not detectable in any urine sample from him after dermal exposure and his recorded urine volume was questionable for calculating the excreted total BPA-d_{16}. Thus, I only plot his urinary total BPA-d_{16} (µg/g creatinine) after dietary exposure.

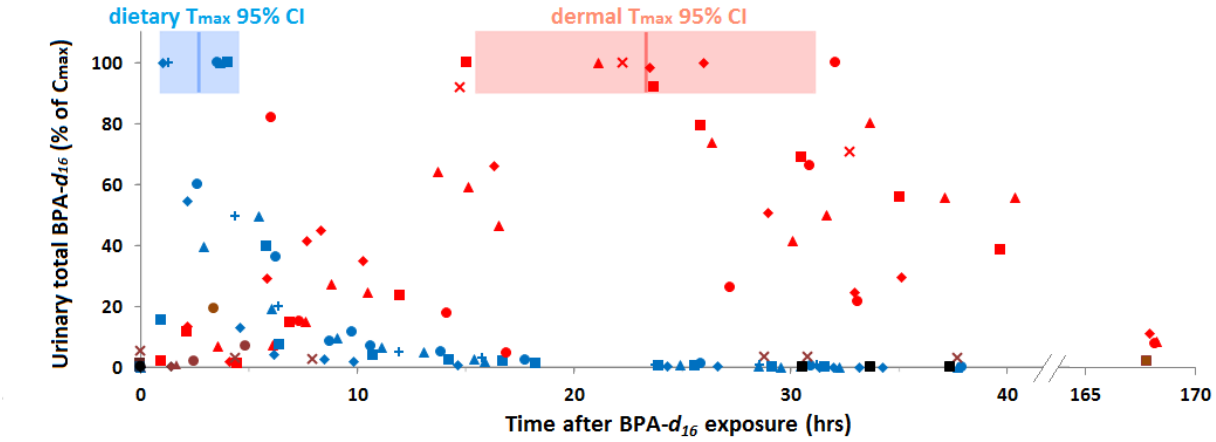

Figure 3-3 Urinary total BPA-d_{16} concentrations following a single dietary exposure (blue) and single dermal contact event (red). Concentrations are creatinine adjusted and plotted by participant (P1 ( ), P2 (■), P3 (▲), P4 (●) P5 (×) and P6 (+)) as percent of maximum concentration for each participant's samples (% of C_{max}). Most data are for the 41 hr post exposure period, while data between 165 and 170 hrs are 'control' urine samples collected one week after the dermal exposure, immediately before the oral exposure study . Blue and red shaded regions represent the mean (vertical line) and 95%CI of T_{max} In the dietary and dermal exposure studies, respectively. Non-detects in the dietary exposure study (in black) and dermal exposure study (in brown) are also colour coded.

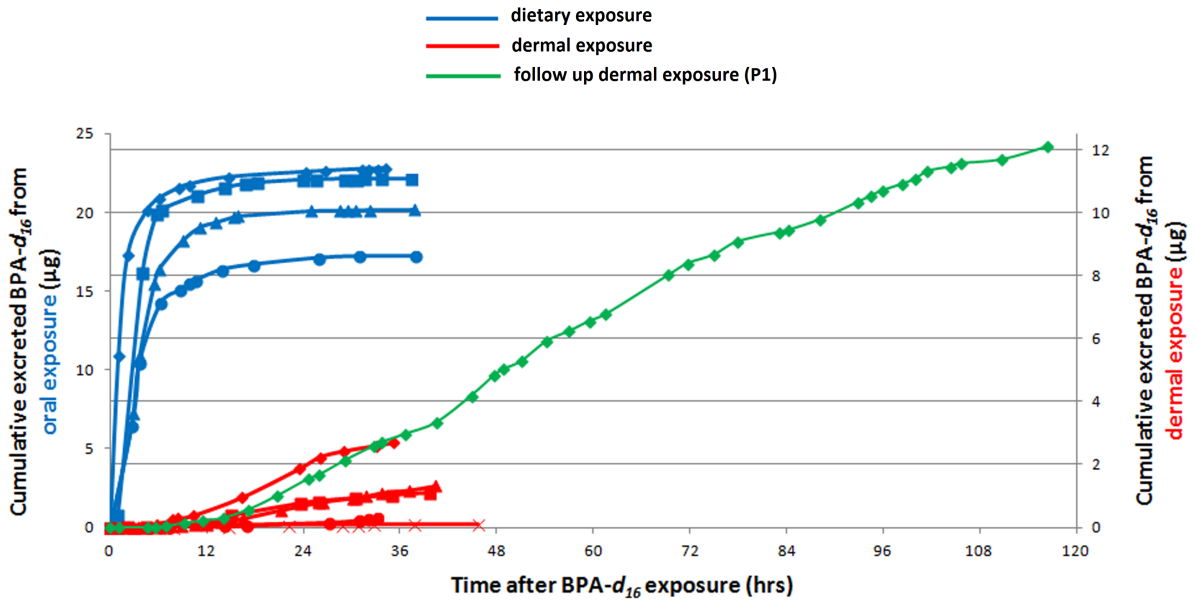

Figure 3-4 Cumulative excreted mass weight of total BPA-d_{16} in participants after oral exposure and dermal exposure for P1 ( ), P2 (■), P3 (▲), P4 (●) and P5 (×).

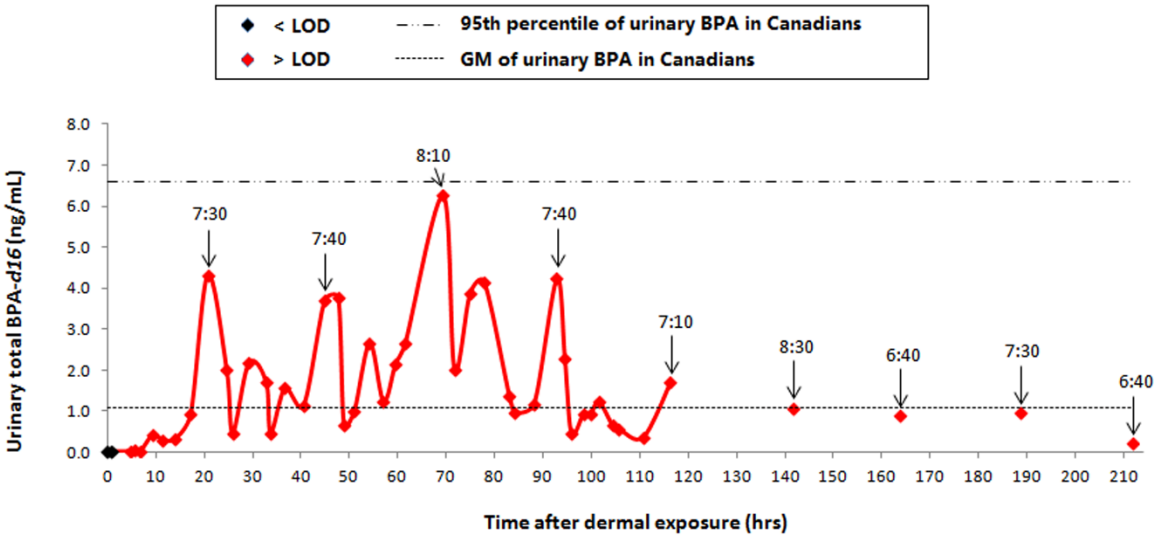

Figure 3-5 Urinary concentration of total BPA-d_{16} (ng/mL) for one participant over 9 days after handling a simulated receipt paper (25 mg BPA-d_{16} /g paper). All urine events were collected in the first 5 days (116 hr) and first morning urine samples were then collected to day 9. First morning urine events are indicated by arrows and time of collection. The geometric mean (GM) and 95th percentile of urinary BPA concentration from Canadian national survey[4] are also plotted in the figure.

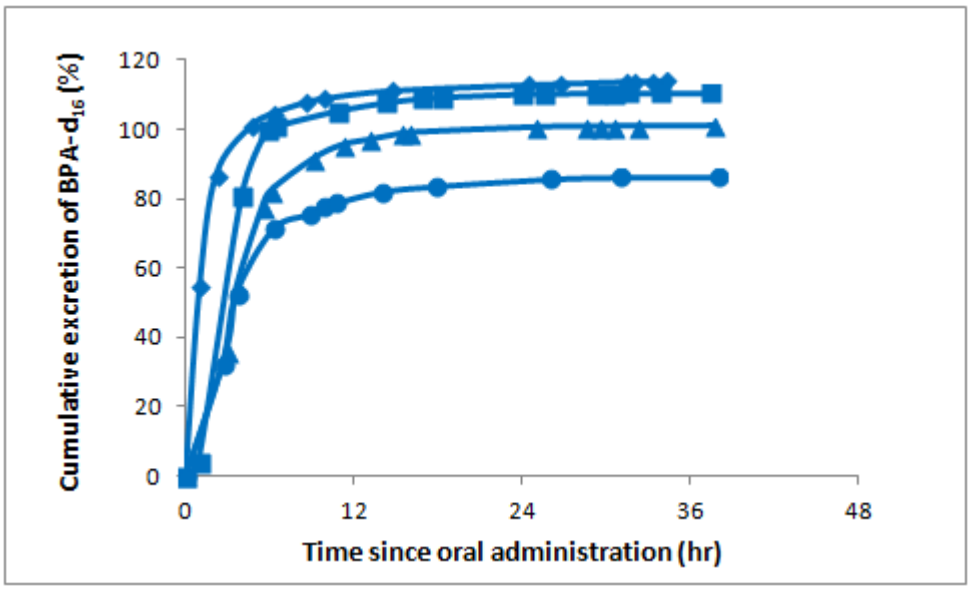

Figure 3-6 Cumulative excreted percent of total BPA-d_{16} dose orally administrated to each participant (n=4).

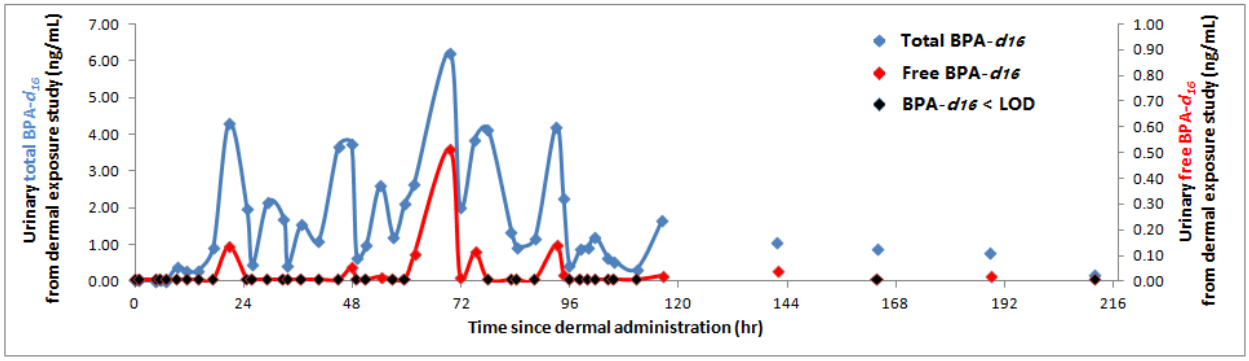

Figure 3-7 Urinary total and free BPA-d_{16} within 9 days after dermal exposure.

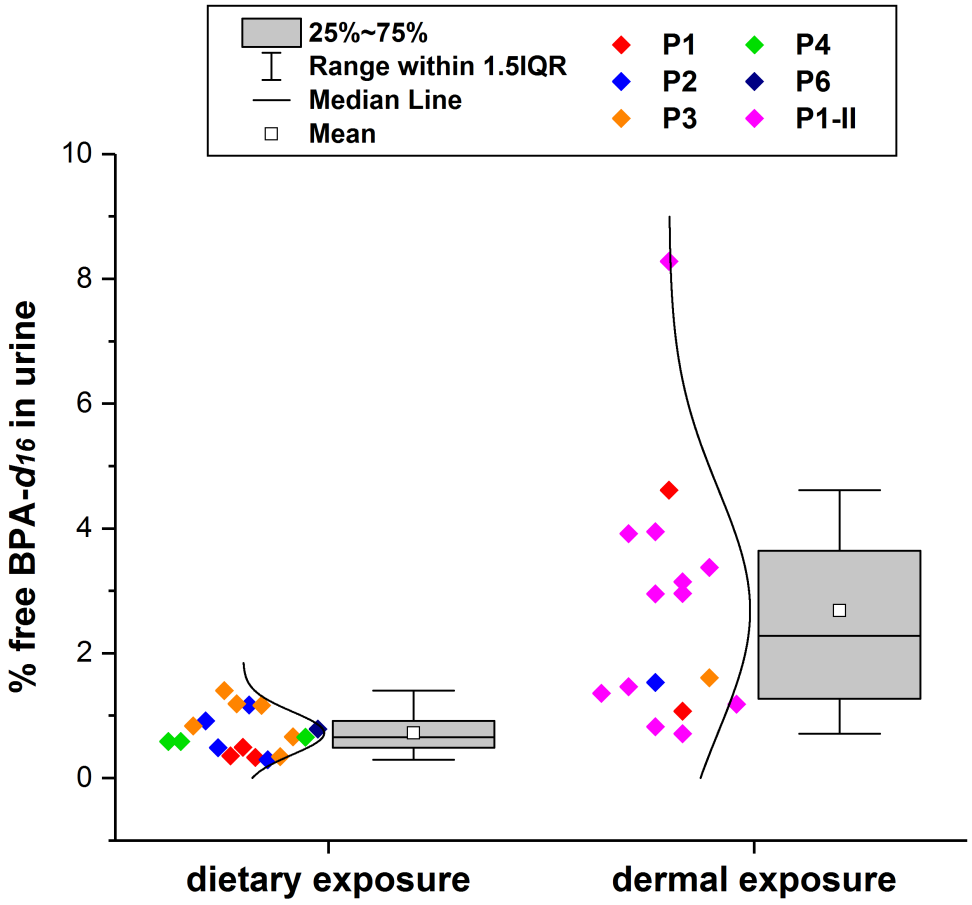

Figure 3-8 Box plot showing free BPA-d_{16} concentration as a percent of total urinary BPA-d_{16} following dermal exposure (16 results from 3 participants) and dietary exposure (17 results from 5 participants). Data indicated as P1-II are for P1 in the follow up dermal exposure study. Raw data and distribution curves are shown on the left of each box.

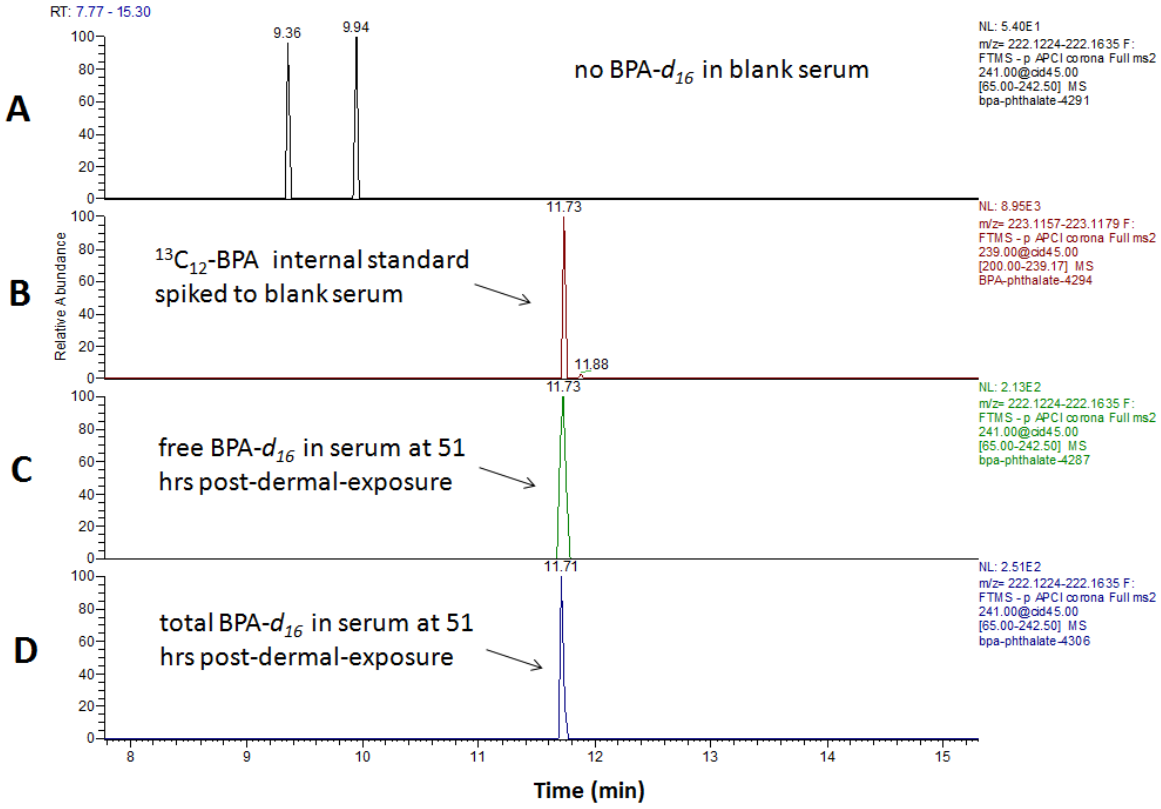

Figure 3-9 HPLC-Orbitrap extracted ion chromatograms of blank serum, showing no traces of BPA-d_{16} (A), 0.50 ng/mL $^{13}C_{12}$-BPA internal standard (for quantification) spiked into blank serum (B), and post-dermal exposure samples showing 0.025 ng/mL free BPA-d_{16} (C) and 0.030 ng/mL total BPA-d_{16} (D) detected in a serum sample from P1, at 51 hrs post-dermal-exposure.

4 Exposure and Dietary Sources of BPA and BPA-Alternatives among Pregnant Women in the APrON Birth Cohort

4.1 Introduction

BPA is an endocrine disruptor that is still widely used in polycarbonate plastics and epoxy resins in food cans, in thermal paper receipts and in dental sealants.[1–3] National surveys in the US and Canada have shown that urinary concentration of BPA was detectable (i.e., >0.2 or >0.4 ng total BPA/mL) in >90% of the general population.[4,5] Owing to increased scientific scrutiny of BPA, and existing bans on its use in certain consumer products (such as baby bottles in Canada,[6] BPA-alternatives are now being used in many commercial products.[7,8] Compared to BPA, these alternative chemicals have similar molecular sizes and structures, and may offer no advantages with respect to endocrine-disrupting activities,[9–11] aquatic toxicity,[12] persistence [12,13] or bioaccumulation potential.[12]

BPS is one such BPA-alternative that has been found in food [14] and paper products.[15,16] Human biomonitoring has shown that BPS was detectable in 81% of urine samples from the USA and seven Asian countries,[17] and its urinary concentrations in American adults increased between 2000 and 2014.[18] BPF was more frequently detected, and at higher concentrations than BPS.[18] BPAF has also been detected in human urine, but with a lower detection frequency (<3%).[18] Sex-specific effects of exposure to BPA on neurodevelopment were observed in animal models [19–22] and also in epidemiological studies.[23] A limited number of animal studies have evaluated the effects of perinatal BPS exposure on neurodevelopment,[24–26] and less is known about the effects of other BPA-alternatives. Given the unique vulnerability of pregnant women and their fetuses,[27] as well as the possibility of inefficient detoxification and clearance of these BPA-analogues in the fetus,[28] it is important to understand the magnitude of exposure and exposure sources for BPA-alternatives in pregnant women.

Diet has been regarded as the major human exposure pathway to BPA.[29–32] BPA in food packaging, especially epoxy resins in cans, can migrate into food.[14,33] A positive association between canned food consumption and urinary BPA concentrations was reported among people in the USA and in European countries.[34–37] A Canadian total diet study (2008–2012) also showed a high concentration of BPA in canned food, while concentrations were low or below LOD in most non-canned food.[38] To our knowledge, no study has yet evaluated the association between diet and urinary concentration of BPS. In contrast to BPA, the concentration of BPS was much lower in foodstuffs and no significant differences in concentrations were found between canned food and other packaged food in Albany, NY, USA in 2008,

2011 and 2012.[14] Nevertheless, BPS was higher in meat and meat products compared to other food categories in this American study, as well as in China in 2012.[14,39]

Following oral exposure, BPA is efficiently metabolized to polar conjugates (>99%) and rapidly cleared through urine with a biological half-life of less than 6 hrs.[40,41] Free BPA, the most toxic form of BPA,[42] comprises less than 1% of total BPA in blood and urine following oral exposure.[40,41] Thus, total BPA in urine has generally been used for biomonitoring of exposure to BPA in humans. However, a recent study reported that the biological half-life is longer when exposure is by the dermal pathway, which may also lead to higher proportions of free BPA in systemic circulation compared to dietary exposure.[43] Therefore, detecting both free and total forms of BPA and BPA-alternatives in human biofluids might provide valuable information for risk assessment of these chemicals.

The present study investigated free and/or total BPA, BPS, BPF and BPAF in 467 second trimester maternal urine samples, and in 455 paired postpartum urine samples from participants enrolled in the Alberta Pregnancy Outcomes and Nutrition (APrON) birth cohort study, Alberta, Canada. The 24 hr intakes were estimated from urinary concentrations and 24-hr dietary recall data were used to estimate dietary sources that influence exposure in this background population of pregnant women.

4.2 Methods

I analyzed the target analytes in maternal urine samples and conducted the data analysis.

4.2.1 Study population.[⑦]

Participants were enrolled during pregnancy to the APrON birth cohort study between 2010 and 2012. Recruitment was previously described [44] and all research protocols were approved by the Health Research Ethics Boards at the Universities of Alberta (Study ID: Pro00002954) and Calgary (Ethics ID: REB14-1702_REN3). Participants were informed of the nature and purpose of the study and provided written informed consent before providing biological samples or completing questionnaires. For the present study, I excluded women who self-identified as smokers. Participants who were recruited after 18 weeks of gestation were excluded, because biological samples from early in the pregnancy were desired.

[⑦] The APrON team was responsible for the participants recruitment.

Samples in the current study were from 467 women who provided urine samples during the second trimester of their pregnancy, and from 455 (97%) of the same women who provided urine samples at three months postpartum. Participants were mainly well-educated, > 30 years of age, Caucasian, and had relatively high family incomes. Of the 467 women recruited during pregnancy, thirty-five did not provide 24-hr food recall data, and 63 had missing covariates. Thus, final models for dietary exposure sources included 369 participants in the second trimester of pregnancy, but these women (n=369) had similar characteristics compared to the overall population (n=467) (Table 4-1).

4.2.2 Data collection.[⑧]

Questionnaires were administered to collect sociodemographic information including maternal age, body weight, height, education, family income, ethnicity and parity. The date and time of urine collection were recorded by staff at the clinic. The 24-hr dietary recall questionnaire was originally designed to assess nutrient intake, thus detailed information including food brand, package type, method of cooking and serving size was recorded for the 24 hr period prior to collection of samples in the second trimester of pregnancy.

4.2.3 Urine collection.[⑨]

Spot urine samples were collected from each participant in the second trimester and at three months postpartum. Sterile urine cups were used to collect urine, which were immediately aliquoted into 9 mL cryovials and stored at -80 °C. Potential contamination of target analytes from the sampling and storage was tested using pure water (HPLC grade) as a surrogate for urine during sample collection, storage and analysis (n=20 control samples). No free BPA or BPA-alternatives were detectable in any of these control samples.

4.2.4 Analytical method.

Total (conjugated plus free bisphenol) and free urinary concentrations of BPA, BPS, BPF and BPAF were quantified by online solid-phase extraction coupled to HPLC–high resolution mass spectrometry (Orbitrap Elite, Thermo Fisher Scientific, San Jose, CA). For detection of total bisphenols (BPs), a 400 µL aliquot of urine was mixed with 10 µL of 1 ng $^{13}C_{12}$-BPA and 200 µL of 1 M ammonium acetate buffer containing 1 µL aqueous solution of β-glucuronidase and sulfatase (≥100,000 units/mL; Sigma, St. Louis,

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[⑧] The APrON team was responsible for the data collection.
[⑨] The APrON team was responsible for the sample collection.

MO). The mixture was incubated at 37 °C overnight to deconjugate the metabolites, and 390 µL of 1 M formic acid was added to achieve 1mL final volume. For analysis of free BPs, the other aliquot (400 µL) was mixed with 10 µL of 1 ng $^{13}C_{12}$-BPA, 200 µL of water and 390 µL of 1 M formic acid without the enzyme deconjugation step. The instrumental method was described in detail.[45] As shown in Table 4-2, recoveries of target analytes at two levels (2.5 ng/mL and 12.5 ng/mL) ranged from 95–116%, with relative standard deviation (RSD) ranging from 3% to 12%. Linearity was valid between 0.5 ng/mL and 50 ng/mL, and regression coefficients of standard curves (6 point curve) were always > 0.99. Urine samples with concentrations above 50 ng/mL were diluted and injected again for accurate quantification. Aliquots of the same samples (1-mL) were sent to the Clinical Trials Laboratory, Alberta Health Services (Edmonton, Alberta), for analysis of creatinine. The LOD of creatinine was 10 mg/dL in urine, and the interday RSD of duplicate injections (n=48) of one urine sample over 4 months was 2%.

4.2.5 Statistical analysis[⑩]

Statistical analyses were performed with Stata software (Stata 12.0; College Station, TX) and SAS (version 9.4; SAS Institute, Cary, NC), and the level of significance for all statistical tests was 0.05. Non-detect concentrations were assigned the arbitrary value of LOD/√2 for certain statistical tests. Non-detectable BPF concentrations were assigned zero for calculation of estimated 24 hr intake of $\sum \mathrm{BPs}$. The t-test, Chi-square test and Cochran-Armitage trend test were used to compare continuous, categorical and ordinal characteristics between overall and included participants, respectively. A paired t-test was used to compare exposure and 24 hr intake in the second trimester to 3 months postpartum. Simple linear regression was used to analyse associations between urinary concentrations of BPA and BPS, as well as between exposure in the second trimester and postpartum. Simple and multiple linear regressions were used on natural log-transformed urinary BPA and BPS concentrations (µg/g creatinine) to evaluate associations between urinary concentrations and sociodemographic factors, consumption of canned food, and consumption of any meat and meat products, respectively. Beta coefficients in these models were exponentiated to the ratio of urinary GM of BPA or BPS of each category with respect to the urinary GM of the reference group; the GM ratio was used previously for evaluating associations between BPA exposure and canned food consumption.[37] Based on previous studies,[34,35,46–48] maternal age, parity, education, family income, ethnicity and daily sampling time were initially included as covariates. Due to multicollinearity among education level, maternal age and family income, only family

[⑩] Dr. Irina Dinu assisted with the data analysis.

income was included in final regression models. I also repeated all analyses excluding extreme values of creatinine (<0.3 g/L or >3.0 g/L) to evaluate the influence from these extreme values (48 of 369 results) on the findings.

4.3 Results and discussion

4.3.1 Total BPA and total BPA-alternatives in second trimester urine

Total BPA was detectable in 93% of second trimester maternal urine with a GM of 1.2 ng/mL. These concentrations were comparable to Health Canada national biomonitoring (2012–2013) where detection frequency was 92% and the GM was 1.1 ng/mL.[4] The most frequently detected BPA-alternative was BPS, detected in 59% of second trimester urine samples. Nevertheless, total BPS concentrations (GM=0.16 ng/mL) were approximately an order of magnitude lower than for total BPA. Interestingly, the maternal urine sample containing highest total BPA (Cmax=44 ng/mL) also contained the highest total BPS (Cmax=240 ng/mL). Moreover, Cmax of total BPS was 5 times higher than Cmax of total BPA (Table 4-3). The homogenous distribution of urinary BPA and BPS concentrations among different sociodemographic groups is shown by overlapping confidence intervals (CI) among groups (Table 4-4).

Total BPF was only detectable in 9% of maternal urine samples, but the 95th percentile (7.3 ng/mL) and Cmax (390 ng/mL) of total BPF equalled or exceeded the concentrations of BPS or BPA (Table 4-3). The low detection frequency of BPF is in part due to the high LOD for BPF (1.0 ng/mL) in the current study, which was 3-10 times higher than the LOD of other analytes in our method (0.10 ~ 0.32 ng/mL) and higher than the LOD of BPF (0.1 ng/mL) in previous work.[18] Nevertheless, in this previous study in the USA,[18] the 95th percentiles of urinary total BPF (6.6 ng/mL) and total BPS (1.3 ng/mL) in urine collected in 2013 (n=141) were comparable to the present study (95th percentile: 7.3 ng/mL for BPF; 1.2 ng/mL for BPS). Thus, exposure to BPF may be comparable to BPS in the current cohort.

Total BPAF was only detected in one second trimester urine sample at a relatively low level (0.20 ng/mL), and with no detectable free BPAF. Total BPAF was also not detected (<0.02~0.44 ng/mL) or only detected in a few samples in previous studies in the USA, China and Saudi Arabia.[18,49] Due to the low detection frequency of BPAF, I did not evaluate the daily intake or dietary sources for this BPA-alternative.

4.3.2 Total BPA and BPS urinary concentrations three months post-partum

Log transformed concentrations of urinary BPA in the second trimester were significantly (p<0.01) but weakly correlated (r^2=0.04) (Figure 4-1) with concentrations 3 months postpartum for the same participants (n=455), suggesting some similarities in exposure sources during pregnancy as after pregnancy for these mothers. The weak association is reasonable due to the short biological half-life of BPA following oral exposure (<6 hrs).[40,41] Notably, BPA concentrations three months postpartum were also significantly lower than in the second trimester (p<0.01) (Table 4-3), perhaps related to excretion of BPA through breast milk.[50,51]

A significant association was also observed for urinary concentration of BPS between the two time points (p<0.01), but the association was even lower (r^2=0.02) (Figure 4-1) and concentrations were comparable during and after the pregnancy. For BPF, the 95th percentile and maximum level were lower postpartum, and the 95th percentile of BPF (4.2 ng/mL) was still comparable to the 95th percentile of BPA (5.0 ng/mL) postpartum. The Cmax of BPF (110 ng/mL) was also much higher than BPA (55 ng/mL) or BPS (72 ng/mL).

4.3.3 Free BPA and free BPA-alternatives in the second trimester

Urinary concentration of free BPA and free BPA-alternatives were monitored in the second trimester but not postpartum. This was because among 467 second trimester urine samples, only 3 had detectable free BPA (Table 4-3). Nevertheless, it is important to note that for these three samples the free BPA, as a proportion of total BPA (14%, 28%, 67%), was higher than in pharmacokinetic studies of human oral exposure (<1.0%) [41] and dermal exposure (<8.3%).[43] Health Canada's national biomonitoring program monitored free BPA in first-trimester urine samples from 1879 pregnant women across Canada between 2008 to 2011 , and reported that the proportion of free BPA was up to 12% (95th percentile) and the maximum proportion of free BPA was 63%.[52] No free BPA was detected in the one urine sample with the highest total BPA (44 ng/mL), suggesting that the proportion of free BPA was less than 0.7% in this sample.

Free BPS was detected in 9 of 467 second trimester urine samples, and the highest free BPS concentration (1.5 ng/mL) was detected in the urine sample with the highest total BPS concentration (Cmax=240 ng/mL) (Figure 4-2); the proportion of free BPS was 0.6%. Similarly, only one urine sample had detectable free BPF (2.0 ng/mL) and this was detected in the sample with the highest total BPF concentration (Cmax=390 ng/mL) (Figure 4-2); the proportion of free BPF was 0.5%.

Overall, the low detection frequency of free BPA and BPA-alternatives supports the efficient metabolism of these phenols in humans, and furthermore demonstrates effective quality control in our

sample collection, storage and laboratory analysis. For BPA, the percentage of free BPA to total BPA in urine was less than 1.0% following oral exposure in humans [40,41] and was less than 8.3% following dermal exposure,[43] but to our knowledge no study has yet evaluated the pharmacokinetics of BPS and BPF in humans. The current result is suggestive that the efficiency of BPS and BPF biotransformation is similar to BPA in humans.

4.3.4 Estimated 24 hr Intake of BPA, BPS and BPF in the second trimester and 3 months postpartum

The estimated 24 hr intake (ng/kg BW/d) of BPA, BPS and BPF were calculated based on creatinine output and assuming that approximately 100% of daily intake is excreted through urine within 24 hrs.[40,41,53] This method is previously described,[36,54,55] but briefly the calculation used was:

$$EDI = UC_{crea} \times CE_{smoothed} / BW \quad (1)$$

where UC_{crea} is the urinary creatinine-adjusted concentration of BPA, BPS and BPF (μg/g creatinine), BW is body weight (kg) in the second trimester or 3 months postpartum, and $CE_{smoothed}$ (g/day) is the 24-hr urinary creatinine excretion calculated the following way:

$$CE_{smoothed}(\mu g/d) = 1.64 \times (140 - age) \times BW^{1.5} \times height^{0.5} \quad (2)$$

where 1.64 and 140 are constants,[54] and age (yrs), body weight (kg) and height (cm) are for each participant at the specific time point. This calculation was used for estimating the daily intake of BPA in females in a previous study,[36] but it did not consider the changes in urinary creatinine excretion during pregnancy and the prolonged BPA exposure following dermal exposure. Thus, the estimated results should be interpreted with caution.

For BPA, the GM of estimated 24 hr intake was 32 ng/kg BW/d in the second trimester, and 21 ng/kg BW/d postpartum (Table 4-5). These values are in the range of previous estimates of dietary exposure in Canada for females (ages 19-30, 42 ng BPA/kg BW/d) based on a *Total Diet Study* and recorded food intakes.[56] This result is not inconsistent with dietary exposure being the major exposure source of BPA for our participants. As with urinary concentrations, the estimated 24 hr intake of BPA in the second trimester was significantly higher than that postpartum for our participants (p<0.01), and again no significant difference in concentration of urinary BPS was observed at the two time points. The maximum and 95th percentile of estimated 24 hr intake for BPA, BPS and BPF were also higher in the second trimester compared to that at 3 months postpartum (Table 4-5).

The maximum estimated 24 hr intakes of BPA (1.1 μg/kg BW/d in second trimester, 0.74 μg/kg BW/d postpartum) were lower than the provisional TDI of 25 μg/kg BW/d established by the Food Directorate

of Health Canada [57] or the latest TDI (4 µg/kg BW/d) established by the European Food Safety Authority (EFSA).[58] To our knowledge, there are no established TDI's for any BPA-alternative, but because of their similar structure and toxicities [9–12] I compared the estimated 24 hr intake (in molar concentration) of BPS and BPF to the TDI of BPA. The 95th percentile 24 hr intakes of BPS and BPF (Table 4-5) were also lower than the BPA TDI's set by Health Canada (110 nmol/kg BW/d) [57] or EFSA (18 nmol/kg BW/d).[58] However, the maximum estimated 24 hr intake of BPS in the current work (14 nmol/kg BW/d, second trimester) was close to the BPA TDI set by EFSA [58] (Figure 4-3). Moreover, for BPF the estimated 24 hr intake of four pregnant women in the second trimester (range 21 – 30 nmol/kg BW/d) and one woman at 3 months postpartum (23 nmol/kg BW/d) exceeded the EFSA BPA TDI of 18 nmol/kg BW/d [58] (Figure 4-3).

Assuming concentration addition, and summing the estimated 24 hr intakes of BPA, BPS and BPF, estimated 24 hr intake of $\sum \mathrm{BP}$ for four pregnant women (21 – 30 nmol/kg BW/d) in the second trimester were higher than the EFSA BPA TDI (Table 4-5, Figure 4-3). The relatively high exposure to BPA-alternatives in a small proportion of pregnant women points to the need to understand the exposures sources, so that mitigation measures can be undertaken to lower the risk of these BPA-alternatives for protection of all Canadians.

4.3.5 Dietary exposure sources of BPA and BPS

Relatively high concentrations of BPA have been detected in canned food from grocery stores in Canada,[38] thus I first evaluated the contribution of canned food consumption to BPA and BPS exposure in maternal urine using second trimester 24 hr dietary recall data. Participants were grouped based on whether they reported ingesting canned food (vegetables or fruit, soup, meat or pasta) in the 24 hrs prior to sample collection. Due to the low detection frequency and concentration of BPA in canned beverages in the *Canadian Total Diet Study* (2008-2012),[38] canned beverages were not included in this category. Overall, creatinine-adjusted urinary total BPA was significantly higher (22%) in the canned food exposure group than in the non-canned food group (GM ratio: 1.22; 95% CI 1.02, 1.45) (Table 4-6).

In subanalysis, participants who ate more than one type of canned food in the preceding 24 hrs had an even higher significant difference (77%) relative to the reference group (GM ratio: 1.77; 95% CI 1.25, 2.51). Among types of canned food, the greatest significant difference (GM ratio, 95% CI) was for those who had consumed canned meat (1.86, 95% CI 1.24-2.79), followed by those consuming canned vegetables and fruit (1.47, 95% CI 1.09-1.98). Those who had consumed canned soup or canned pasta did not have significantly elevated urinary concentration of BPA compared to the reference group (Table 4-6). This is somewhat different from the Canadian Total Diet Study (2008–2012), in which

concentrations of BPA were higher in canned soup compared to many other food categories.[38] This could be due to the food weight and the meal time of the consumed canned food, which I could not adjust for in the analysis. Nevertheless, the results of both the Canadian Total Diet Study and the present study suggest that canned food is a major exposure source of BPA to Canadian women during pregnancy.

After controlling for parity, family income, ethnicity and urine sample collection time, the significant associations between creatinine-adjusted urinary concentration of total BPA and canned food consumption did not change (Table 4-6). Furthermore, after excluding extreme creatinine values from the data analyses, the positive association between canned food consumption and urinary BPA concentrations did not change (Table 4-7). Using 24-hr dietary recall data from the National Health and Nutrition Examination Survey in the USA, Hartle et al. also found a positive association between canned food consumption and urinary BPA concentrations.[37]

Concentrations of BPA were significantly correlated with BPS in both pre- and postpartum urine samples, but the association was weak (r^2 = 0.03) (Figure 4-1). This result suggests only minor common exposure sources for these two phenols, and this is further supported by the fact that, unlike for BPA, urinary concentration of BPS (GM: 0.22 μg/g) was not significantly different among those consuming canned foods in the preceding 24 hours, relative to those not consuming canned food (GM: 0.23 μg/g). Moreover, no significant differences were found for urinary concentration of BPS between any specific canned food consumption group and the reference group (Table 4-6). No previous study that I am aware of has evaluated the concentrations of BPS in Canadian foodstuff, but Liao et al. found that BPS concentrations in canned food were not higher than in other foods packaged in glass, paper or plastic in the USA.[14]

Based on a survey of BPS in foodstuff collected from retail grocery stores in Albany, NY (USA) in 2008, 2011 and 2012, and from nine cities in China in 2012,[14,39] BPS was much higher in meat and meat products (mean BPS: 0.61 ng/g in USA, 2.2 ng/g in China) than in other food groups (range of mean: 0.005–0.040 ng/g in USA, 0.005–0.64 ng/g in China). Thus, in the current study I grouped participants based on whether they ate any meat or meat products (excluding fish and seafood) in the 24 hrs preceding sample collection. Urinary concentration of BPS (μg/g creatinine) was not significantly different among participants who consumed meat or meat products relative to the reference group that did not eat meat, and no significant difference was observed even when considering participants who

ate more than one meat food item, or who ate >150 g meat, or >200 g meat (Table 4-6). Similar non-significant results were found for BPA and meat consumption (Table 4-6).

Thus, dietary exposure sources of BPS in this Canada cohort are not as important as they are for BPA. BPS is frequently detected in indoor dust [59] and has been detected at high levels on thermal receipt paper and other paper products.[15,16,60] I collected 20 thermal receipts in local stores in Edmonton, Canada, 2015, and found that 9 of 20 receipts contained mainly BPS (Table 4-8). The EFSA previously estimated that dermal exposure of BPA from thermal paper contact contributed approximately 10% of total BPA exposure for women of ages 18 – 45.[61] For BPS, where daily exposure was calculated to be approximately an order of magnitude lower than for BPA in present study, dermal exposure might play a more important role. However, only occupational exposures have evaluated the dermal exposure of BPS to date for humans. For example, a study in cashiers reported a significant increase in urinary total BPS following work shifts.[60] More studies are needed to evaluate non-dietary exposure sources of BPS, particularly considering that dermally absorbed BPA has a much longer half-life compared to dietary absorption.[43]

Dietary exposure sources of BPF could not be rigorously evaluated because of the low detection frequency of urinary BPF (9%). Nevertheless, among participants who had detectable urinary BPF in the second trimester (n=35), 40% consumed canned food the day before the sample collection. This can be compared to 44% of participants consuming canned food in the previous 24 hrs who did not have detectable urinary BPF (n=334). Notably, the one participant with Cmax of urinary BPF also did not consume any canned food in 24 hrs preceding the sample collection. No study that I am aware of has evaluated the concentration of BPF in Canadian foodstuff, however food analysis from the USA in 2008, 2011 and 2012 showed that the concentrations of BPF in canned food was not higher than in other packaged food.[14] These results suggested that canned food consumption is not a predictor of high BPF exposure. Other dietary exposure sources and non-dietary exposure sources of BPF should be evaluated.

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Table 4-1 Comparison of characteristics among APrON participants overall who provided a urine samples in the second trimester and participants included in the final model for evaluating dietary exposure sources of BPA or BPS (n=369).

Characteristics	Participants overall	Participants included	*p*-value
Parity			0.93
Nulliparous	249 (54%)	199 (54%)	
Multiparous	208 (46%)	170 (46%)	
Maternal age (years)			
	32 (±4.1)	32 (±4.1)	0.93
Education			0.71
Primary	4 (0.88%)	3 (0.81%)	
High school	28 (6.1%)	23 (6.2%)	
College	78 (17%)	66 (18%)	
University	223 (49%)	182 (49%)	
Post-graduate	124 (27%)	95 (26%)	
Family income			0.64
<20,000	10 (2.2%)	9 (2.4%)	
20,000–39,999	20 (4.4%)	17 (4.6%)	
40,000–69,999	51 (11%)	45 (12%)	
70,000–99,999	102 (22%)	80 (22%)	
≥100,000	275 (60%)	218 (59%)	
Ethnicity			0.99
Caucasian	401 (87%)	322 (87%)	
Asian	35 (7.6%)	27 (7.3%)	
Latin American	13 (2.8%)	11 (3.0%)	
Others	10 (2.2%)	9 (2.4%)	
Daily sampling time (hrs)			
	14 (±3.2)	14 (±3.1)	0.99

Values are n (%), or mean (±SD). The *p*-value indicates differences between overall and included participants using the *t-test* for continuous variates, *chi-square* for categorical variates, and *Cochran-Armitage trend test* for ordinal variates.

Table 4-2. Mass transition, limit of detection (LOD), recoveries and relative standard deviation of target analytes.

Compounds	Mass transition or Molecular weight	LOD (ng/mL)	Recovery[a] (2.5ng/mL)	Recovery[a] (12.5ng/mL)
BPA	249>211.0766	0.32	105%±7%	109%±11%
$^{13}C_{12}$-BPA	239>223.1168	-	-	-
BPS	249.0227	0.10	116%±9%	103%±10%
BPF	199.0754	1.0	97%±4%	95%±3%
BPAF	335.0501	0.10	105%±11%	111%±12%

[a] n=5.

Table 4-3. Target analyte geometric mean (GM), percentiles (P10-P95) and maximum concentrations in maternal urine collected in the second trimester (n=467) and in paired samples at three months post-partum (n=455).

Compounds	Unit	%>LOD	GM (95% CI)	P10	P25	P50	P75	P95	Max
Second trimester urine samples (n=467)									
Total BPA	ng/mL	93%	1.23 (1.11, 1.36)	0.30	0.59	1.19	2.34	8.20	44.0
	µg/g		1.68 (1.55, 1.83)	0.59	0.88	1.56	2.80	9.57	98.5
Total BPS	ng/mL	59%	0.16 (0.15, 0.18)	NC	NC	0.12	0.28	1.16	243
	µg/g		0.22 (0.20, 0.25)	NC	NC	0.19	0.39	1.58	192
Total BPF	ng/mL	9%	NC	NC	NC	NC	NC	7.29	390
	µg/g		NC	NC	NC	NC	NC	8.04	264
Free BPA	ng/mL	3/467	NC	NC	NC	NC	NC	NC	1.35
	µg/g		NC	NC	NC	NC	NC	NC	1.27
Free BPS	ng/mL	9/467	NC	NC	NC	NC	NC	NC	1.49
	µg/g		NC	NC	NC	NC	NC	NC	1.18
Free BPF	ng/mL	1/467	NC	NC	NC	NC	NC	NC	2.00
	µg/g		NC	NC	NC	NC	NC	NC	1.16
Three months postpartum urine samples [a] (n=455)									
Total BPA	ng/mL	89%	0.95 (0.87, 1.04)	<LOD	0.48	0.91	1.68	4.97	54.6
	µg/g		1.12 (1.04, 1.21)	<LOD	0.60	1.03	1.92	4.92	41.6
Total BPS	ng/mL	64%	0.17 (0.16, 0.19)	NC	NC	0.13	0.27	1.30	72.1
	µg/g		0.20 (0.18, 0.23)	NC	NC	0.17	0.38	1.31	67.2
Total BPF	ng/mL	9%	NC	NC	NC	NC	NC	4.24	114
	µg/g		NC	NC	NC	NC	NC	4.08	185

LOD: limit of detection; NC: not calculated due to the low detection frequency
GM: geometric mean
95% CI: confidence interval of GM
P10: 10^{th} percentile; P25: 25^{th} percentile; P50: 50^{th} percentile; P75: 75^{th} percentile; P95: 95^{th} percentile;
Max: maximum value;
µg/g: µg/g creatinine.

Table 4-4 Associations between sociodemographic characteristics and maternal urinary BPA and BPS concentrations (μg/g creatinine) in the second trimester (n=369).

Characteristic	n	BPA		BPS	
		GM (95% CI)	Adjusted GM ratio (95% CI)	GM (95% CI)	Adjusted GM ratio (95% CI)
Parity					
Nulliparous	199	1.80(1.59, 2.04)	1.16 (0.96, 1.40)	0.23 (0.20, 0.27)	0.97 (0.77, 1.22)
Multiparous	170	1.55(1.35,1.79)	reference	0.24 (0.20, 0.29)	reference
Maternal age					
<25	11	1.52 (0.87, 2.66)	reference	0.17 (0.08, 0.38)	reference
25-29	100	1.63 (1.35, 1.96)	1.07 (0.60, 1.92)	0.22 (0.17, 0.28)	1.26 (0.59, 2.69)
30-34	173	1.71 (1.50, 1.95)	1.12 (0.66, 1.92)	0.25 (0.21, 0.29)	1.42 (0.70, 2.89)
≥35	85	1.71 (1.39, 2.10)	1.12 (0.62, 2.04)	0.25 (0.20, 0.31)	1.43 (0.74, 2.77)
Education					
Primary	3	3.29 (1.92, 5.63)	NA[b]	0.57 (0.03, 10.2)	NA[b]
High school	23	1.33 (0.93, 1.90)	reference	0.24 (0.15, 0.39)	reference
College	66	1.45 (1.18, 1.85)	1.11 (0.72, 1.71)	0.23 (0.16, 0.33)	0.96 (0.48, 1.89)
University	182	1.70 (1.48, 1.95)	1.28 (0.86, 1.92)	0.23 (0.20, 0.27)	0.97 (0.60, 1.55)
Post-graduate	95	1.87 (1.57, 2.23)	1.41 (0.95, 2.09)	0.24 (0.20, 0.29)	0.99 (0.63, 1.57)
Family income					
<20,000	9	1.49 (0.65, 3.40)	reference	0.25 (0.11, 0.54)	reference
20,000–39,999	17	1.65 (1.18, 2.31)	1.11 (0.55, 2.24)	0.23 (0.12, 0.43)	0.93 (0.35, 2.47)
40,000–69,999	45	1.29 (0.99, 1.67)	0.86 (0.45, 1.67)	0.28 (0.18, 0.42)	1.11 (0.41, 3.04)
70,000–99,999	80	1.81 (1.47, 2.25)	1.22 (0.62, 2.41)	0.26 (0.20, 0.34)	1.04 (0.45, 2.39)
≥100,000	218	1.74 (1.54, 1.96)	1.17 (0.64, 2.15)	0.22 (0.19, 0.25)	0.89 (0.44, 1.81)
Ethnicity					
Caucasian	323	1.69 (1.53, 1.86)	reference	0.24 (0.21, 0.27)	reference
Asian	26	1.52 (1.04, 2.22)	0.90 (0.63, 1.29)	0.21 (0.14, 0.33)	0.90 (0.56, 1.42)
Latin American	11	1.58 (0.70, 3.57)	0.94 (0.54, 1.63)	0.22 (0.13, 0.37)	0.93 (0.46, 1.86)
Others	9	2.20 (1.55, 3.13)	NA[b]	0.34 (0.17, 0.71)	NA[b]

[a] *p*-Value for overall group effect.

[b] not calculated because of the small sample size in this group.

Table 4-5 Maternal estimated 24 hr intake of BPA, BPS, BPF and $\sum BPs$ in second trimester and three months postpartum.

Compounds	unit	GM (95% CI)	P10	P25	P50	P75	P95	Max	n>TDI[a]
Estimated 24 hr intake assessment from second trimester urine samples									
BPA	ng/kg BW/d	32 (29, 35)	11	17	30	54	180	1,100	0
	nmol/kg BW/d	0.14 (0.13, 0.15)	0.050	0.076	0.13	0.24	0.73	4.7	0
BPS	ng/kg BW/d	4.2 (3.8, 4.7)	NC	NC	3.8	7.8	30	3,500	0
	nmol/kg BW/d	0.017(0.015,0.019)	NC	NC	0.015	0.031	0.12	14	0
BPF	ng/kg BW/d	NC	NC	NC	NC	NC	160	6,000	4
	nmol/kg BW/d	NC	NC	NC	NC	NC	0.81	30	4
$\sum$ BPs [b]	nmol/kg BW/d	0.21 (0.19, 0.23)	0.067	0.10	0.18	0.34	1.7	30	4
Estimated 24 hr intake assessment from three months postpartum urine samples									
BPA	ng/kg BW/d	21 (20., 23)	NC	12	20	34	84	740	0
	nmol/kg BW/d	0.094(0.087,0.10)	NC	0.050	0.087	0.16	0.38	3.7	0
BPS	ng/kg BW/d	3.9 (3.5, 4.4)	NC	NC	3.3	7.3	27	1,400	0
	nmol/kg BW/d	0.016(0.014,0.018)	NC	NC	0.014	0.030	0.11	4.8	0
BPF	ng/kg BW/d	NC	NC	NC	NC	NC	100	4,600	1
	nmol/kg BW/d	NC	NC	NC	NC	NC	0.51	23	1
$\sum$ BPs [b]	nmol/kg BW/d	0.15 (0.13, 0.16)	0.048	0.069	0.12	0.24	1.4	23	1

NC: not calculated due to low detection frequency; GM: geometric mean; 95% CI: confidence interval of GM; P10: 10th percentile; P25: 25th percentile; P50: 50th percentile; P75: 75th percentile; P95: 95th percentile; Max: maximum value.

[a] TDI of BPA from the European Food Safety Authority (4 μg/kg body weight/day; 18 nmol/kg body weight/day).

[b] sum of estimated 24 hr intake of BPA, BPS and BPF; the estimated 24 hr intake of BPF for non-detects was substituted to be 0 in the calculation.

Table 4-6 Association between maternal urinary BPA or BPS concentrations (μg/g creatinine) in the second trimester with canned food, and meat and meat product consumption.

		BPA		BPS	
		Unadjusted[a]	Adjusted[b]	Unadjusted[a]	Adjusted
Exposure sources	n	GM ratio (95% CI)	GM ratio (95% CI)	GM ratio (95% CI)	GM ratio (95% CI)
Canned food consumption in 24 hrs prior to sample collection					
Canned food	160	1.22 (1.02, 1.45)*	1.25 (1.04, 1.50)*	0.99 (0.80, 1.23)	0.97 (0.77, 1.23)
>1 canned food	24	1.77 (1.25, 2.51)**	1.84 (1.26, 2.69)**	0.98 (0.63, 1.51)	0.95 (0.59, 1.52)
Canned meat	20	1.86 (1.24, 2.79)**	1.73 (1.13, 2.64)*	0.79 (0.48, 1.31)	0.77 (0.46, 1.30)
Canned vegetables/fruit	37	1.47 (1.09, 1.98)**	1.48 (1.08, 2.02)*	1.15 (0.79, 1.69)	1.13 (0.75, 1.68)
Canned soup	73	1.12 (0.89, 1.41)	1.18 (0.92, 1.51)	1.03 (0.78, 1.37)	0.99 (0.73, 1.35)
Canned pasta	22	0.87 (0.60, 1.27)	1.03 (0.69, 1.53)	1.23 (0.76, 1.98)	1.26 (0.75, 2.10)
No canned food	209	reference	reference	reference	reference
Meat and meat product consumption in 24 hrs prior to sample collection[c]					
Ate meat	299	0.92 (0.72, 1.17)	0.91 (0.71, 1.16)	0.82 (0.60, 1.11)	0.82 (0.60, 1.13)
>1 meat food	121	0.95 (0.71, 1.25)	0.95 (0.71, 1.26)	0.81 (0.57, 1.16)	0.86 (0.60, 1.25)
>150g meat food	113	0.89 (0.66, 1.19)	0.87 (0.64, 1.18)	0.84 (0.58, 1.22)	0.88 (0.60, 1.31)
>200g meat food	69	0.88 (0.63, 1.23)	0.86 (0.61, 1.21)	0.76 (0.51, 1.14)	0.79 (0.52, 1.21)
No meat food	70	reference	reference	reference	reference

[a] Used log transformed creatinine adjusted urinary BPA and did not adjust any covariable.

[b] Adjusted for parity, family income, ethnicity and urine sampling time of day.

[c] Meat here did not include fish and seafood.

*p<0.05, **p<0.01

Table 4-7 Association between maternal urinary BPA or BPS concentrations (μg/g creatinine) in the second trimester with canned food, or meat and meat product consumption, after exclusion of extreme creatinine values from the data analyses.

		BPA		BPS	
		Model 1[a]	Model 2[b]	Model 1[a]	Model 2[b]
Exposure sources	n	GM ratio (95% CI)	GM ratio (95% CI)	GM ratio (95% CI)	GM ratio (95% CI)
Canned food consumption the day before sample collection					
Canned food	139	1.25 (1.03, 1.51)*	1.27 (1.03, 1.56)*	1.05 (0.84, 1.32)	1.03 (0.79, 1.33)
>1 canned food	21	1.78 (1.21, 2.63)**	1.95 (1.30, 2.94)**	0.99 (0.63, 1.55)	0.96 (0.59, 1.57)
Canned meat	19	1.97 (1.29, 3.02)**	1.90 (1.22, 2.94)**	0.89 (0.54, 1.44)	0.84 (0.50, 1.41)
Canned vegetables/fruits	29	1.57 (1.11, 2.23)*	1.57 (1.10, 2.23)*	1.19 (0.79, 1.81)	1.16 (0.75, 1.80)
Canned soup	63	1.10 (0.86, 1.42)	1.19 (0.90, 1.56)	1.09 (0.81, 1.46)	1.03 (0.74, 1.44)
Canned pasta	20	0.98 (0.66, 1.47)	1.08 (0.71, 1.65)	1.36 (0.84, 2.19)	1.42 (0.84, 2.39)
No canned food	182	reference	reference	reference	reference
Meat and meat product consumption the day before sample collection[c]					
Ate meat	258	0.88 (0.69, 1.13)	0.89 (0.69, 1.16)	0.79 (0.59, 1.04)	0.82 (0.60, 1.12)
>1 meat food	108	0.97 (0.72, 1.29)	0.96 (0.71, 1.30)	0.86 (0.62, 1.20)	0.94 (0.64, 1,36)
>150 g meat food	100	0.91 (0.66, 1.24)	0.84 (0.60, 1.16)	0.84 (0.60, 1.17)	0.90 (0.62, 1.31)
>200 g meat food	63	0.98 (0.69, 1.41)	0.92 (0.63, 1.33)	0.84 (0.56, 1.25)	0.86 (0.56, 1.34)
No meat food	63	reference	reference	reference	reference

[a] Used log transformed creatinine-adjusted urinary BPA and did not adjust any covariable.

[b] Adjusted for parity, family income, ethnicity and urine sampling time of day.

[c] Meat here did not include fish and seafood.

*p<0.05, **p<0.01

Table 4-8 Concentration of BPA and BPS on 20 thermal receipts from stores in Edmonton, Canada.

Thermal Receipt #	BPA (mg/g)	BPS (mg/g)
R1	**32.33**	0.04
R2	0.03	**9.36**
R3	**31.38**	0.03
R4	**39.30**	0.01
R5	**14.83**	0.02
R6	0.03	**12.89**
R7	**26.42**	0.06
R8	**19.25**	ND[a]
R9	0.02	**5.60**
R10	0.03	**86.96**
R11	0.03	**35.26**
R12	0.02	**77.66**
R13	0.04	**54.80**
R14	**28.39**	ND[a]
R15	0.03	**16.80**
R16	ND[a]	**17.94**
R17	**21.93**	0.03
R18	**17.02**	ND[a]
R19	**23.04**	ND[a]
R20	**14.11**	ND[a]

[a] not detectable

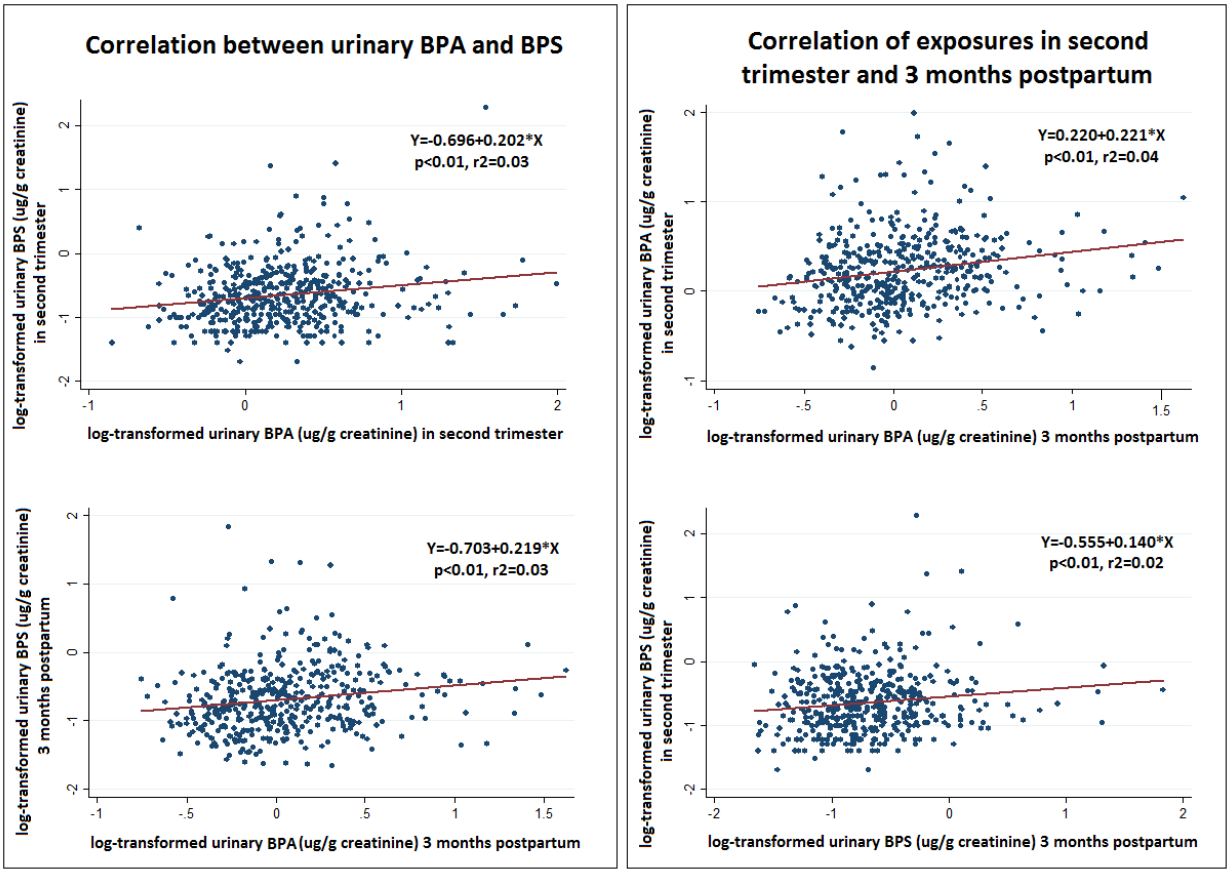

Figure 4-1 Correlation between log transformed creatinine adjusted urinary BPA and BPS, and their exposure levels between second trimester and postpartum.

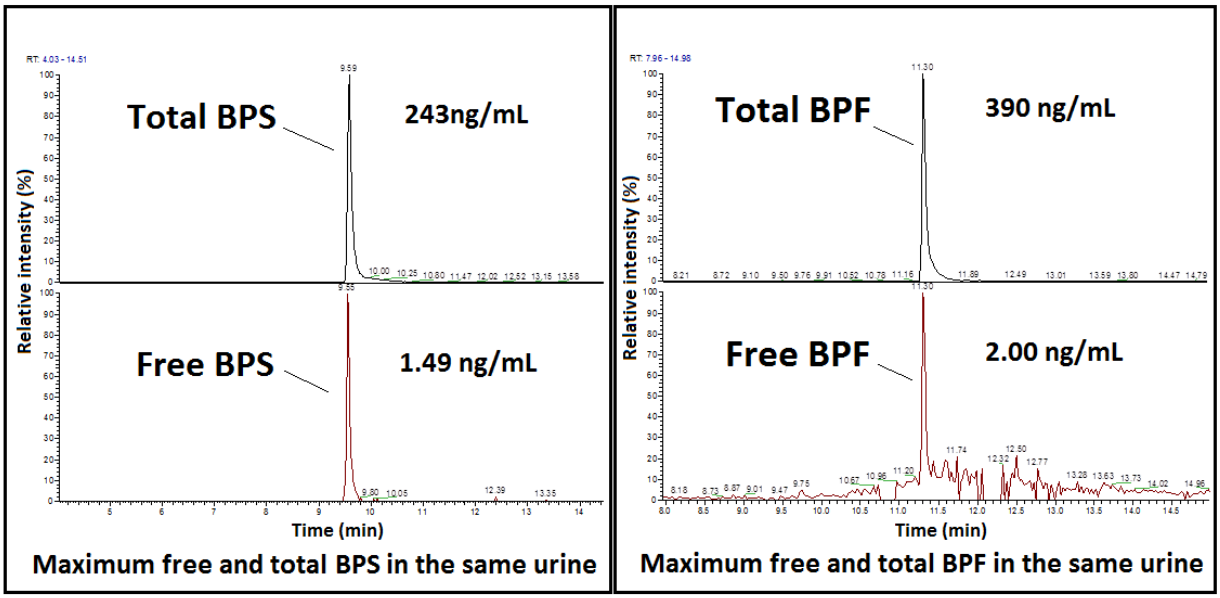

Figure 4-2 Chromatogram of maximum free BPS (1.49 ng/mL) and total BPS (240 ng/mL) detected in the same second trimester urine sample. Chromatogram of maximum free BPF (2.00 ng/mL) and total BPF (390 ng/mL) detected in the same second trimester urine sample.

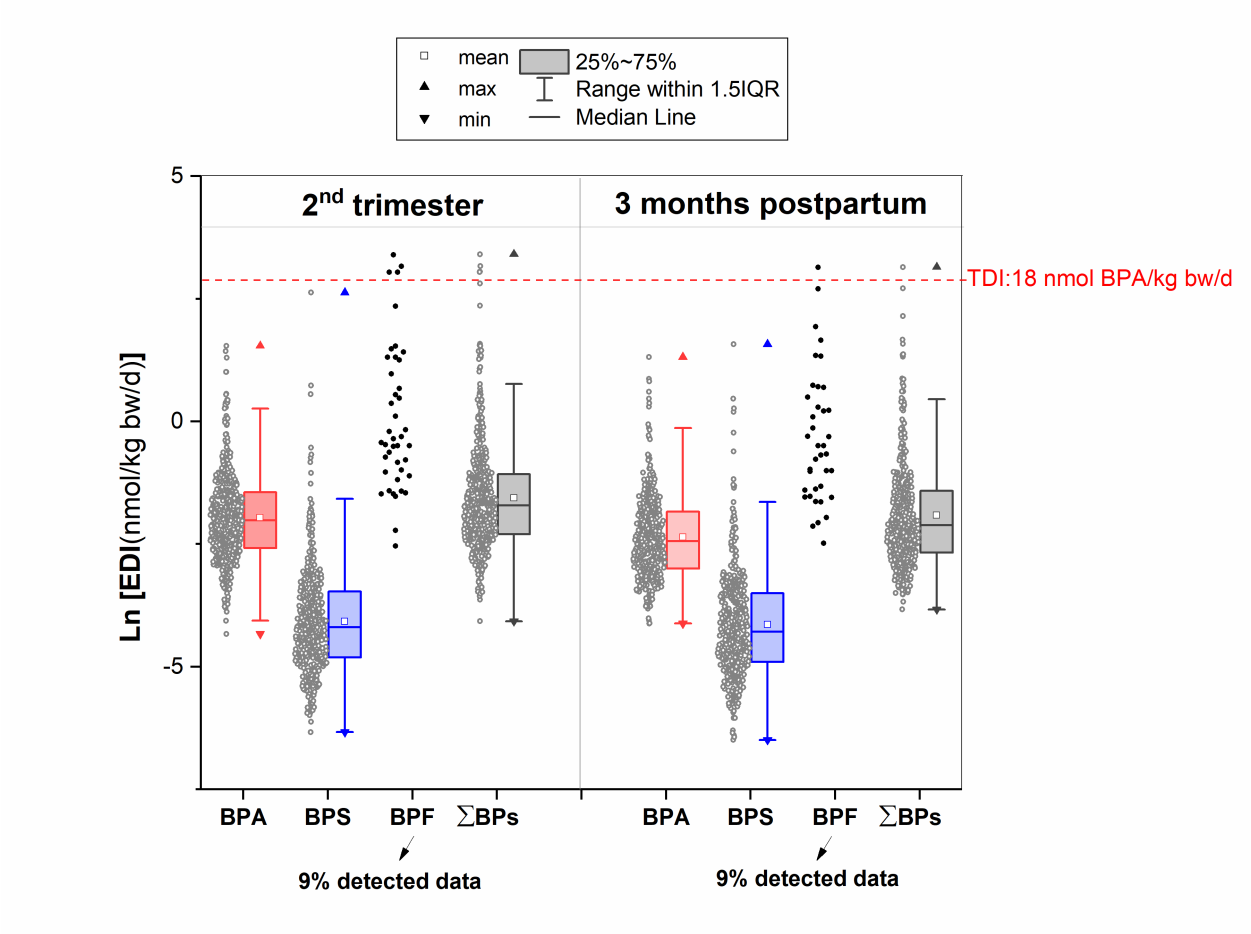

Figure 4-3 Box plot of estimated 24 hr intake (EDI) of total BPA, BPS, BPF and $\sum BPs$ calculated from creatinine adjusted concentration in urine collected in second trimester and 3 months postpartum. The original data are showed on the left of each box plot. As only 9% of urine samples have detectable total BPF, only original data of detected BPF are shown in the figure. The non-detects of BPF is substituted to zero for calculating the EDI of $\sum BPs$. The TDI of BPA from the European Food Safety Authority (4 μg/kg body weight/day) is marked by dashed line.

5 Interaction of Maternal Nutrients and Bisphenol Exposures on Neurodevelopment and Behaviour of 2 Year-Olds

5.1 Introduction

BPA is a endocrine disruptor widely used in polycarbonate plastic manufacturing, epoxy resins, and commercial products such as food and liquid containers, children's toys and medical devices.[1] The primary source of human exposure is thought to be leaching from food and drink containers, leading to widespread human exposure via ingestion.[2] BPA is frequently detectable in human biofluids, according to national surveys in Canada (2012-2013) and in the USA (2003-2012); approximately 90% of the general population have detectable urinary concentration of BPA (i.e. >0.2 or >0.4 ng total BPA/mL).[3,4]

After oral exposure, free BPA (the toxic form of BPA) is efficiently detoxified through biotransformation to metabolites that are rapidly excreted through urine, [5,6] and the biological half-life for BPA is less than 6 hrs in adults.[5,6] However, the biotransformation capacity, especially glucuronidation capacity of the human fetus, is immature,[7] and deconjugation of BPA-glucuronide back to free BPA was observed in rat fetuses.[8] Total BPA has been detected in cord serum collected in Germany,[9] the USA,[10] and Canada.[11] One study reported higher BPA metabolites in cord serum than in maternal serum, suggesting slower clearance of BPA metabolites in the fetus than in the mothers.[12] Pregnancy is a critical window of exposure due to rapid and critical developmental processes occurring in the fetus, and subtle effects during this period can lead to functional deficits and increased disease risk later in life,[13] consistent with the general hypothesis of the fetal origins of health and disease.[14] Thus, there is an emerging public health concern about the possible adverse effects of widespread maternal and perinatal BPA exposure.

In animal studies, perinatal exposure to BPA can cause physical alterations in the developing brain, such as decreased cells in cortical plate and midbrain dopamine neurons, as well as behavioural changes, including social and anxiety-related behaviours in juvenile and adult animals, but with the observed effects being variable with dosage and sex.[15] For example, perinatal exposure to BPA (3 μg/g food) significantly decreased the density of tyrosine hydroxylase-immunoreactive neurons in substantia nigra of female mice, but no significant effects were observed in male mice or at a higher exposure (8 mg/g food).[16] Ryan et al. reported that prenatal and early postnatal oral exposure to BPA (2 and 200 μg/kg bw/day by gavage) increased anxious behaviours in ovariectomized female mice three weeks after weaning in a dose-dependent manner, but did not evaluate the effects on males.[17] Another study

reported that neonatal exposure to BPA (50 μg/kg bw/day by subcutaneous injection) increased adult anxiety and aggression in male rats, but female rats were not tested.[18] Gioiosa et al. evaluated the effects of perinatal BPA exposure on behaviour of female and male mice at an exposure level within the range of humans (10 μg/kg bw/day through oral pathway).[19] Their results indicated that sex differences were decreased in BPA-exposed animals with respect to explorative and emotional behaviours, such as novelty-seeking levels and anxiety.[19]

As reviewed by Ejaredar et al., associations between prenatal BPA exposure and behaviour in children were observed in epidemiology studies, but reported sex-specific effects differ among studies.[20] For example, prenatal BPA exposure was positively associated with externalizing problems in girls at 2 years of age,[21] and anxious and depressed behaviour among girls at age 3,[22] while the associations were null or negative among boys in these studies. In contrast, Perera et al. reported that higher prenatal BPA exposure was associated with significantly more problems in emotional reactivity and aggression among boys between 3 and 5 years of age, compared to fewer problems of anxious/depressed and aggressive behaviour among girls.[23] The varying sex-specific effects associated with prenatal BPA exposure may be due to genetic differences between study populations, unexamined co-exposures or uncontrolled covariates related to neurodevelopment of children.[24]

Exposure to BPA is mainly through the diet [25–27] which is also an important exposure source of classic neurotoxicants. For example, high levels of heavy metals, such as cadmium and arsenic, are detected in canned fish,[28] which is also an important exposure source of BPA.[29,30] Any adverse effects caused by co-exposure to such neurotoxic metals in the diet could therefore confound the relationship between BPA exposure and neurodevelopment of children. Maternal dietary intake of micronutrients also affects fetal growth and has the potential to impact future behaviour and cognitive function.[13,31] BPA's ability to disrupt DNA methylation (i.e., hypomethylation) at certain gene loci [32–35] is regarded as a possible mechanism affecting brain development and behaviour [15] and has been shown to be counteracted by supplementation of nutrients involved in DNA methylation (i.e. folic acid, choline, vitamin B12 and betaine) in animal studies.[32,33] To our knowledge, previous epidemiological studies of BPA exposure have not adjusted for co-exposures to dietary neurotoxic heavy metals, nor to maternal nutrient status.

Due to increasing scientific scrutiny and regulatory restrictions on BPA, alternatives such as bisphenol S (BPS) have being used in a number of consumer product applications.[36] Biomonitoring shows that BPS is detectable (i.e., >0.02 ng/mL) in 81% of urine samples collected in China, the USA, and in six other Asian countries.[37] Urinary concentrations of BPS are much lower than BPA in the general population,[37,38]

but use and exposure are increasing.[38] In embryonic zebrafish, low-dose BPS treatment resulted in a 240% increase in hypothalamic neurogenesis, which was greater than the effect caused by BPA (180% increase) at the same dose.[39] However, no epidemiologic studies have yet reported on prenatal BPS exposure and neurodevelopment of children.

Here I examined the association between prenatal exposure to BPA and BPS, and neurodevelopment and behaviour problems in children at age 2. The confounders adjusted in the models were selected from a pool of covariates including four heavy metals that are established developmental neurotoxicants,[40] and prenatal maternal status of micronutrients including three methyl donating nutrients: vitamin B12, choline, and folate. The effect measure modification of child's sex and maternal status of nutrients involved in DNA methylation were evaluated in this study.

5.2 Methods

I was responsible for the participant selection, detection of bisphenols in maternal urine samples and data analysis.

5.2.1 Study population and samples.

All biological samples and data were from mothers and children participating in the APrON study.[11] From 2009 to 2012, 2140 pregnant women residing in Calgary and Edmonton (Alberta, Canada) were recruited into this longitudinal birth cohort study.[41] All protocols for APrON and this sub-study were reviewed and approved by health research ethics boards at the Universities of Alberta (Study ID: Pro00002954) and Calgary (Ethics ID: REB14-1702_REN3) and participants provided informed consent at time of recruitment and prior to neurodevelopmental testing of children. Participating mothers completed questionnaires at time of recruitment to provide data on sociodemographics, including maternal age, ethnicity, education level and family income at time of recruitment. Sex and gestational age at birth (weeks) of children were obtained from hospital birth records.

For the current study, I included mothers who provided maternal urine samples at the second trimester (13 – 28 weeks of gestation), based on the study of Braun et al. who reported that early gestational exposure to BPA (16 weeks) was more strongly correlated with child behavioural outcomes

[11] APrON team was responsible for the participants recruitment, data collection and sample collection.

than later prenatal exposure (i.e. 26 weeks).[22] I also limited inclusion to mothers who reported to be non-smokers or quitting smoking during pregnancy, resulting in 467 qualified pregnant women (Figure 5-1). I further excluded children who had genetic disorders associated with deficits in intellectual or motor ability (n=7). For the multivariable data analysis, I excluded participants who were missing maternal nutrient data (n=47), or who did not provide all required sociodemographic data (n=17). Finally, 394 eligible mother-child pairs presented for neurodevelopmental testing [the Bayley Scales of Infant Development – Third Edition (Bayley-III)] and 358 completed the Child Behaviour Checklist (CBCL) at age 2.

5.2.2 BPA and BPS trace analysis.

Total BPA and BPS were quantified in spot maternal urine samples collected at the time of the 2nd trimester APrON interview. The average gestational time [±standard deviation (SD)] of urine sample (n=467) collection was 17 (±1.7) weeks. Sterile urine cups were used to collect urine samples, which were immediately aliquoted into 9 mL cryovials and stored at -80 °C. The potential for contamination of target analytes during sampling or storage materials was tested by using HPLC grade water as a surrogate matrix for urine during the process of sample collection, storage and analysis (n=20). Briefly, no BPA or BPS were detected in these quality control blanks.

The total concentrations of BPA and BPS (i.e. conjugated plus unconjugated) were quantified by online solid-phase extraction coupled to high performance liquid chromatography and an Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA). An aliquot of 400 µL urine was mixed with 10 µL of internal standard (1 ng $^{13}C_{12}$-BPA) and 200 µL of 1 M ammonium acetate buffer containing 1 µL aqueous solution of β-glucuronidase and sulfatase (≥100,000 units/mL, Sigma, St. Louis, MO). The mixture was incubated at 37°C overnight to deconjugate any metabolites, then 390 µL of 1 M formic acid was added to bring the sample to 1mL final volume. The instrumental method has been described,[42] and recoveries of target analytes at two levels (2.5 ng/mL and 12.5 ng/mL) ranged from 103 - 116%, with RSD ranging from 7% to 11%. A quality control sample (1ng/mL target analytes spiked in a blank urine) was analyzed with each batch (10 to 50) of urine samples, and the RSDs of the repeated analyses (n=39) over 10 months were 11% for BPA and 16% for BPS. The LODs for the method were 0.32 ng/mL for BPA and 0.10 ng/mL for BPS. Linearity was excellent ($r^2 > 0.99$) over two orders of magnitude (0.5 ng/mL to 50 ng/mL, 6 point curve) and any urine sample with a concentration higher than 50 ng/mL was diluted by LC grade water and re-injected for accurate quantification.

A 1 mL aliquot of each maternal urine sample was also sent to the Clinical Trials Laboratory (Alberta Health Services, Edmonton, Alberta) for quantitative analysis of creatinine. The reported LOD of creatinine was 10 mg/dL in urine. For quality control I submitted the same reference sample with each batch of APrON samples, and the RSD of repeated analyses (n=48) over 4 months was 2%.

5.2.3 Neurodevelopmental and behavioural outcomes.[12]

5.2.3.1 Neurodevelopmental assessment.

The neurodevelopmental assessments were conducted between 2013 and 2015 by psychometrists in the Behavioural Research Unit at Alberta Children's Hospital. The psychometrists were supervised by a registered psychologist and were trained on the Bayley-III. The assessments were completed in dedicated assessment rooms. The Bayley-III is the most widely used measure of neurodevelopment in young children.[43] It includes five distinct scales: Cognitive (items assessing sensorimotor development, memory and other aspects of cognitive processing), Language (receptive communication and expressive communication items), Motor (fine motor and gross motor items), Social Emotional (child's interactions with peers and adults, ability to explain what they need and why, ability to describe how they feel and ability to use emotions in an interactive, purposeful manner) and Adaptive Behaviour (communication, community use, functional pre-academics, home living, health and safety, leisure, self-care, self-direction, social and motor). The composite scores from each of these five scales were used.

5.2.3.2 Child Behaviour Checklist; CBCL.

The CBCL is a well-validated parent-report questionnaire that assesses child behaviour problems with good reliability, stability and validity.[44] Parents, typically the mother, completed the CBCL when their children were 2 years old. The 99-item CBCL was completed in English. The CBCL comprises seven syndrome scales including Emotionally Reactive, Anxious/Depressed, Somatic Complaints, Withdrawn, Sleep Problems, Attention Problems and Aggressive Behaviour. Two composite scales are derived from the syndrome scales: Internalizing Problems (the sum of Emotionally Reactive, Anxious Depressed, Somatic Complaints and Withdrawn) and Externalizing Problems (the sum of Attention Problems and Aggressive Behaviour). As recommended by the manual of the CBCL,[44] raw scores calculated for each syndrome scale were used.

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[12] Dr. Deborah Dewey and her team were responsible for the neurodevelopmental and behaviour assessment.

5.2.4 Potential confounders and effect measure modifiers.

5.2.4.1 Heavy metals.

Heavy metals in maternal red blood cells collected during second trimester were analyzed at the Alberta Centre for Toxicology (University of Calgary) where an inductively coupled plasma – triple quadrupole mass spectrometer was used to detect arsenic (As), cadmium (Cd), lead (Pb) and total mercury (Hg). The method has been described,[45] and LODs are shown in Table 5-1.

5.2.4.2 Maternal nutrients.[13]

Copper (Cu), zinc (Zn), manganese (Mn) and selenium (Se) were analyzed in the same maternal red blood cells and using the same method as for heavy metals. Using other aliquots of whole blood, plasma, serum and red blood cells from mothers in the second trimester, the team of Dr. Catherine Field analyzed serum ferritin, whole blood hemoglobin, plasma vitamin B12, red blood cell folate, serum docosahexaenoic acid (DHA) and serum arachidonic acid (AA) using analytical methods as previously described.[46] Dietary intakes of choline were estimated in the second trimester from 24 hr dietary recall questionnaires.[47] For a small portion of mothers who had no available data for fatty acids (n=8), choline (n=13), and hemoglobin (n=29), their status of these nutrients in the first trimester (fatty acids) and third trimester (choline and hemoglobin) were used. The status of these nutrients at second trimester were comparable with their status in selected alternate period among around 400 participating mothers without significant difference.

5.2.5 Statistical analysis.[14]

Statistical analyses were performed with SAS (version 9.4; SAS Institute Inc., Cary, NC) and the level of significance was $p<0.05$. Marginal significance ($p<0.10$) was used for selecting the interaction for the final model. Urinary concentrations of BPA or BPS below LOD (8% for BPA, 42% for BPS) were assigned a value of LOD/√2. For heavy metals, ≥ 74% samples had a concentration above LOD (Table 5-1), and reported concentrations in the analysis were used even if the results were < LOD. Possible differences in demographic characteristics, urine collection times, and prenatal exposure to BPA and BPS between the participants included and excluded (Figure 5-1) were tested by *Chi-square tests* for categorical variables, by the *Cochran-Armitage trend tests* for ordinal variables, and by *independent t-tests* for continuous

[13] Dr. Catherine Field and her team was responsible for evaluating the maternal nutrients.
[14] Dr Irina Dinu and Dr. Leah J. Martin assisted the data analysis.

variables. The *independent t-test* was also used to compare the maternal nutrients status in the second trimester and selected alternate period (first trimester for fatty acids; third trimester for choline and hemoglobin), assess the sex-specific difference in prenatal exposure of BPA and BPS, as well as Bayley-III and CBCL scores. The paired *t*-test was used to compare prenatal exposure of BPA to BPS.

5.2.5.1 Outcome variables.

Composite scores from Bayley-III assessments were normally distributed, thus *linear regression* was used to analyze these scores as five separate, continuous outcomes. Raw scores for the seven CBCL syndrome scales, which are the sums of the children's scores on each individual item, were right-skewed, thus *Poisson regression* was used to analyze these syndrome scales and two composite scales as nine separate outcomes. *Poisson regression* was also used to analyze the relationship between BPA exposure and CBCL scores in a previous study.[23]

5.2.5.2 Independent variables and effect measure modifiers.

Due to the right-skewed distribution of BPA concentrations in urine, the BPA concentrations (creatinine-adjusted) were $\log_{10}$ transformed and analyzed as continuous variables. Given that BPS was only detected in 58% of urine samples, the BPS concentrations (μg/g creatinine) were dichotomized (upper quartile versus lower three quartiles) and analyzed as a categorical variable. High/low BPS exposure was only marginally associated with prenatal BPA exposure (p=0.081), which might be due to the difference in major exposure sources of BPS and BPA in our participants (introduced in Chapter 4).

As potential confounders, I considered the following variables: maternal nutrient status (ferritin, hemoglobin, vitamin B12, choline, folate, copper, zinc, manganese, selenium, DHA and AA), co-exposure to heavy metals (As, Cd, Pb, Hg), child's sex, child's gestational age at birth, child's age at assessment (months), family income, mother's educational level, maternal age at birth of the child and mother's ethnicity. Of these, a variable was included in all models of Bayley-III or all models of CBCL scores if that variable was significantly (p<0.05) associated in bivariable analyses with at least one assessment score of the Bayley-III or the CBCL, respectively. Multicollinearity was tested among covariates by *Pearson correlation* (for two continuous variables), *ANOVA* (for continuous and categorical variables) and *Chi-square test* (for two categorical variables). Due to significant multicollinearity among family income, educational level and age of the mothers, only family income, which was significantly correlated with more outcomes or had higher measures of association, was included in the final models.

I *a priori* planned and tested child's sex and nutrients involved in DNA methylation as potential effect measure modifiers of the associations between the exposures of interest (i.e., BPA and BPS) and each of the five Bayley-III scores and the nine CBCL scores. To evaluate nutrients involved in DNA methylation, I created a dichotomous dummy variable (methyl donors) based on whether maternal status of the three nutrients involved in DNA methylation (vitamin B12, folate and choline) was above the median for each of these three nutrients. Thus, high status of methyl donors refers to the situation where folate, vitamin B12 and choline were all above median (n=56 for Bayley III; n=55 for CBCL), and low status refers to all other cases (n=338 for Bayley III; n=303 for CBCL). Significant interaction (p<0.10) between bisphenol and sex was included in the models for testing the interaction between bisphenol and methyl donor status. I only kept marginally significant interaction terms (p<0.10) in the final models. The three-way interaction (bisphenol × sex × methyl donors) was tested if the interaction terms of bisphenol × sex and bisphenol × methyl donors were all significant (p<0.10). All first order interactions (bisphenol × sex, bisphenol × methyl donors, sex × methyl donors) were included in the model for evaluating the second order interaction (bisphenol × sex × methyl donors). The stratum-specific estimates (95% CI) of the associations for boys and girls, or for children with high and low maternal status of methyl donors, were calculated from the model with all participants (n=394 for Bayley III; n=358 for CBCL) and any marginally statistically significant interaction terms.

5.3 Results

5.3.1 Population characteristics

Among all non-smoking maternal participants who provided a urine sample in the second trimester (n=467), the mothers were mainly Caucasian (88%) and well-educated (93% finished college or higher education), with a median age of 31 (interquartile range = 29-34) and most had an annual family income greater than $70,000 CAD (83%). According to Statistics Canada, median family income for the recruitment region was $94,460 in 2012.[48] Among characteristics of participants included or excluded from the final study populations (Tables 5-2, 5-3), no significant differences were observed with respect to bisphenol exposure, birth record data, maternal demographics or child's sex.

5.3.2 BPA and BPS in maternal urine

Total BPA and BPS were detectable in >90% and 58%, respectively, of maternal urine samples (Table 5-4). Concentrations of BPA [GM= 1.7 µg BPA/g creatinine] were approximately 6 times higher than BPS

(GM = 0.23 µg BPS/g creatinine), and the difference was statistically significant (p<0.01). Log-transformed urinary BPA concentrations (µg/g creatinine) were positively associated with BPS concentrations (p<0.01), but the r-square was very small (r^2=0.03) (Figure 4-1). There were no significant differences in maternal exposure to BPA and BPS among births giving rise to boys compared to girls.

Total BPA concentrations in maternal urine ranged from <0.14 (LOD adjusted by creatinine) to 60 µg BPA/g, indicating a wide range of exposure (approximately 2 orders of magnitude). The relative range of total BPS in maternal urine was even greater, from <0.02 µg/g (LOD adjusted by creatinine) up to 192 µg/g (approximately 4 orders of magnitude).

5.3.3 Maternal status of nutrients and heavy metals.

In addition to child's sex and demographic covariates of mothers, I also considered the potential confounding effect on neurodevelopment and behaviour from co-exposure to heavy metals and maternal nutrients. The four heavy metals examined have been shown to be developmental neurotoxicants and are frequently detected in human samples.[40] The detection frequency of these heavy metals ranged from 74% to 99.6% in maternal red blood cells (Table 5-1), and only a few mothers (n≤2) had an exposure level higher than the reference ranges for these heavy metals. The selected maternal nutrients are known to be positively associated with neurodevelopment in children.[49–52] The children were grouped based on their mother's intake of three nutrients involved in DNA methylation (folate, vitamin B12, choline) and I hypothesized that increasing exposure to these nutrients might mitigate any effect from bisphenols. The percent of the samples with deficient levels of iron, vitamin B12 and folate were very low (Table 5-1), while the proportion of mothers with low levels of Zn and Se were 37% and 29%, respectively; however, no universally agreed deficiency cut-offs were found for these two micronutrients.

5.3.4 Neurodevelopmental assessment

Bayley-III composite scores of boys were significantly lower than those of girls for all five scales (Table 5-5), as has been reported in previous research.[53] Thus, child's sex was included as an confounder in all adjusted models.

In unadjusted analysis and the adjusted analysis including only main effects, prenatal BPA and BPS exposures were not statistically significantly related to the scores for any of the Bayley-III subscales (Table 5-6). For the Social Emotional subscale, I found child's sex to be a statistically significant effect measure modifier of the relationship between BPA and composite scores (Table 5-6). After adjusting for

sex, family income, maternal ethnicity, child age at assessment, maternal nutrients (vitamin B12, folate, copper, manganese, selenium), co-exposure of cadmium, and the interaction between BPA and child's sex, a 1-unit increase in $\log_{10}$-transformed urinary BPA concentrations was negatively associated with composite scores on the Social Emotional subscale for boys (β = -5.1, 95% CI: -9.6, -0.63) (Table 5-6), indicating that boys with higher prenatal exposure to BPA had lower performance on such criteria as: explaining what they need and why, describing how they feel, and using emotions in an interactive, purposeful manner. However, for girls, the stratum-specific association between BPA and Social Emotional composite scores was not statistically significant (β = 3.8, 95% CI: -0.60, 8.3).

The effect measure modification effects of sex were also observed for the relationship between BPS (high vs. low) and Bayley-III subscales of Language (*p*=0.044), with marginally significance on Cognitive (*p*=0.070) and Motor (*p*=0.090) subscales. After adjusting for prenatal BPA exposure and the other covariates mentioned above for the Social Emotional subscale model, higher prenatal BPS exposure (upper quartile) among girls was significantly associated with lower scores on the Motor subscale (β = -4.9, 95% CI: -9.6, -0.20), while no significant stratum-specific association was found for boys (Table 5-6).

Methyl donor status was another potential effect measure modifier I tested for the association between bisphenols and neurodevelopment. The significant or marginally significant interactions between bisphenol and sex were adjusted in the models for testing the modifications of methyl donor status (Table 5-7). For Social Emotional scores, the interaction between maternal methyl donor status (high/low) and BPA was significant in the adjusted model without other interaction terms (Table 5-7). However, after adjusting for the significant effect measure modifier of sex, the modification effect of methyl donors became no longer marginally statistically significant (*p*=0.11). Thus, maternal methyl donor status was not included as an effect measure modifier of the relationship between BPA and the Social Emotional subscale score. The interaction between methyl donor status and BPS was marginally significant for the Cognitive subscale (*p*=0.070), but it became not significant after adjusting the effect measure modification of sex (Table 5-7).

5.3.5 Child behavioural problems.

The distribution of CBCL scores (Table 5-5) showed that boys had statistically significantly higher raw scores (i.e. more problems) on the following syndrome and composite scales: Attention Problems, Aggressive Behaviour, and Externalizing Problems and also tended to have higher scores on the Withdrawn scale (*p*=0.07). Overall, the distribution of CBCL raw scores for Externalizing Problems (mean:

10.3; SD: 7.0) and Internalizing Problems (mean: 5.9; SD: 5.6) were comparable with those of a non-Latino White middle-upper income group included in a previous study in the USA.[54]

In unadjusted analyses, BPA exposure was positively associated with 3 of 7 CBCL syndrome scores, as well as internalizing and externalizing problems, while BPS was only positively associated with Anxious/Depressed syndrome (Table 5-8). Maternal nutrients status (ferritin, vitamin B12, folate, choline, Cu, Zn, Mn, AA, Se), co-exposure to neurotoxic metals (Cd, Hg and Pb), child's sex, family income, maternal ethnicity and gestational weeks at birth were significantly associated with at least one CBCL syndrome score in bivariable analyses; therefore, I adjusted for these variables as potential confounders to estimate relationships between prenatal BPA and BPS exposures and CBCL scores. In adjusted main effects models, significant positive associations were observed between prenatal BPA exposure and 4 of 7 syndrome behaviours, and externalizing problems among all children (Table 5-8). After adjusting for BPA exposure and outcome-related covariates, higher BPS exposure was only associated with more sleep problems (Table 5-8).

The interaction between prenatal BPA exposure and sex was statistically significant for Aggressive behaviour and Externalizing Problems; therefore, I included this interaction term in these models (Table 5-8). In multivariable models that additionally included this interaction term, for girls, stratum-specific BPA exposure was positively associated (p<0.05) with more aggressive behaviour and more externalizing problems. In contrast, for boys, prenatal BPA exposure was not associated with these behaviour syndromes or problems (Table 5-8). Sex was also a significant effect measure modifier of the relationship between BPS and Aggressive behaviour syndrome. In the adjusted model that included this interaction term, for girls, higher BPS exposure was positively associated (p<0.05) with more aggressive behaviour. In contrast, for boys, prenatal BPS exposure was not associated with this behaviour syndrome (Table 5-8).

The modification effects of maternal methyl donor status on relationships between bisphenols and CBCL scores were firstly tested in multivariable models including selected potential confounders and the interaction term of bisphenol × methyl donor status. Significant or marginally significant interaction between BPA and methyl donor status was observed for Anxious/depressed syndrome, Sleep problems Aggressive behaviour and Externalizing problems (Table 5-9). Estimated from this model, BPA exposure was positively associated with more Sleep problems among children with low status of maternal methyl donors, while this association was not observed among children with high status of maternal methyl donors. For Aggressive behaviour and Externalizing problems, the interactions of BPA × sex and BPA ×

methyl donor status were all significant (Table 5-9). Thus, both of these two interaction terms were included in the final models for these two CBCL scores. The results showed that: for girls with low status of maternal methyl donors, stratum-specific BPA exposure was positively associated (p<0.05) with more aggressive behaviour and more externalizing problems. In contrast, for boys with high status of maternal methyl donors, higher prenatal BPA exposure was associated (p<0.05) with fewer aggressive behaviours and fewer externalizing problems (Table 5-11). These results supported our hypothesis that the intake of methyl donors during pregnancy may mitigate the adverse effects of prenatal BPA on behaviour of children. The three-way interaction term BPA × sex × methyl donors was not significant for these two CBCL scores, indicating that the interaction of BPA and methyl donors on Aggressive behaviour and externalizing problems was not significantly modified by children's sex.

The interaction between maternal status of methyl donors and prenatal BPS exposure was only marginally significant for Emotionally reactive syndrome and internalizing problems (p<0.10), while the association between the main effect BPS and these behaviour syndromes was not significant after including the interaction in the models (Table 5-10).

5.4 Discussion

In this longitudinal cohort study, I found that, for children at age 2, prenatal BPA and BPS exposures were negatively associated with neurodevelopment, and positively associated with behavioural problems after adjusting for other covariates. Moreover, some of the adverse associations observed for BPA and BPS were sex-specific or moderated by maternal status/intake of folate, vitamin B12, and choline.

Higher prenatal BPA exposure was significantly associated with poorer social emotional neurodevelopment in boys, as shown by lower scores on the Social Emotional subscale of the Bayley-III. Additional adjustment for maternal methyl donor status as an effect measure modifier of the relationship between BPA and social emotional development was not statistically significant in this model. Among all children, higher prenatal BPA exposure was associated with poorer behavioural outcomes on 3 of 7 CBCL syndrome scales (emotionally reactive, sleep problems, attention problems) and, among girls, higher levels of aggressive behaviour and more externalizing problems. These findings using the CBCL are consistent with results of the HOME cohort study, whereby higher prenatal BPA exposure was associated with externalizing behaviours at age 2 and anxious and depressed behaviour at age 3, especially among girls.[21,22] In contrast, Perera et al. reported significant positive correlation

between prenatal BPA exposure and emotionally reactive and aggressive behaviour of boys between 3 to 5 years of age, but the association became negative among girls.[23] In the studies of Harley et al., and Roen et al.,[55] positive associations between prenatal BPA exposure and anxiety and depression, and internalizing and externalizing problems were also only observed in boys at age 7-9 years.[24] However, the MIREC study showed that prenatal BPA exposure was not associated with aggression, anxiety or depression in children at age of 3,[56] but was correlated with more reported somatization and poorer working memory and planning/organizing skills in boys only.[56] Age of assessment, sample size, covariates available for adjustment, and the time of urine collection among these studies could be possible reasons for the variability in the sex-specific effects noted from study to study.[24,57] For example, prenatal BPA exposure was evaluated in an early stage of pregnancy in the present study (second trimester, average 17 weeks of gestation), in the HOME study (16 weeks of gestation),[21,22] and in MIREC (average: 12 weeks of gestation; range: 5.1 – 15 weeks),[56] whereas Perera et al., Roen et al. and Harley et al. quantified exposure in the third trimester, or averaged early and later prenatal measurements.[23,24,55]

Disruption of epigenetic programming of gene expression during development might be an important molecular mechanism that underlies the neurodevelopmental and behavioural effects of BPA.[15] Nutrients involved in one-carbon metabolism, such as folate, choline and vitamin B12, are known to play important roles in the maintenance of genomic DNA methylation.[58,59] Animal studies have demonstrated that the hypomethylation effects of BPA on DNA are counteracted by supplementation with hypermethylators, such as folic acid, vitamin B12 and choline.[32,33] To our knowledge, the current study is the first in humans to investigate modifying effects of nutrients involved in DNA methylation (methyl donor status) on potential adverse developmental effects of BPA or BPS. I detected statistically significant interactions between BPA exposure and methyl donor status for Aggressive behaviour and Externalizing problems, suggesting that the observed associations between prenatal BPA exposure and behaviour syndromes of children could be modified through maternal status/intake of nutrients involved in DNA methylation (folate, vitamin B12 and choline). In animal studies, epigenetic effects of BPA were found to be sex-specific,[34,60] whereby the effects of BPA on gene expression and DNA methylation are different in males and females. However, I did not find significant second order interaction of BPA × sex × methyl donors in our data. It should be noted that the models for testing the second order interaction (BPA × methyl donors × sex) includes 15 covariates for outcome of Bayley-III and 20 covariates for CBCL. Thus, the sample size (n=394 for Bayley-III; n=358 for CBCL) might not be enough for this test.

Experimental studies have investigated the epigenetic effects of BPA in the brain.[34,35,60,61] Yaoi et al. found that low doses of BPA led to both hyper- and hypomethylation in fetal mouse forebrain.[35] Kundakovic et al. reported sex-specific effects, whereby prenatal BPA exposure in mice resulted in a significant increase in DNA methylation in a region corresponding to exon A of estrogen receptor 1 (Esr1) in male prefrontal cortex, but decreased DNA methylation in exon A of Esr1 in the hypothalamus of female.[34] Prenatal BPA exposure also induced sex-specific DNA methylation alterations in the mouse gene corresponding to brain-derived neurotrophic factor.[60] Taken together, these studies suggest that epigenetic effects of BPA may involve both hypermethylation or hypomethylation, depending on genetic loci and sex. As introduced above, maternal supplementation with nutrients involved in DNA methylation, such as folic acid, modified the effects of prenatal BPA exposure on DNA hypomethylation and counteracted the subsequent changes in coat colour of viable yellow agouti mice [32] and impairment of intestinal digestion and absorption functioning in pigs.[33] Although it is not yet clear how methyl donors modify BPA-related effects on neurodevelopment, animal studies show that methyl donors are essential for maintenance of the brain's epigenomic landscape [62,63] and can affect fear, anxiety and memory in mice.[64,65] Furthermore, postnatal supplementation with methyl donors (choline, betaine, folate and vitamin B12) increased total DNA methylation in the brain and reversed the deleterious effects of maternal separation on depression-like behaviour in rats.[66] More epidemiological studies examining nutrient-toxicant interactions are needed to confirm the results I observed in the present study, and animal studies evaluating the combined effects of BPA exposure and methyl donors on genome-wide epigenomic alterations in the brain would help to understand the molecular mechanisms underlying the observed associations BPA's effects, and the protective effects of methyl donors.

To our knowledge, the current data are the first epidemiological evidence for the effects of prenatal BPS exposure on neurodevelopment of children. BPS was only detectable in 58% of urine samples, but after dichotomizing the exposure category and adjusting for prenatal BPA exposure and other covariates, highest prenatal exposure to BPS (upper quartile, compared to lower quartiles) among girls was significantly associated with poorer motor performance (Table 5-6) and more aggressive behaviours (Table 5-8), while for all children it was significantly associated with more sleep problems. Significant interactions between prenatal BPS exposure and child sex were consistently detected on measures of child development (Bayley-III scores) and behaviour syndromes (CBCL scores).

Consistent with the sex-specific effects of BPA, for BPS, sex was found to be a statistically significant effect measure modifier of the relationship between BPS and aggressive behaviour, whereby higher

prenatal bisphenol exposure was significantly associated with more aggressive behaviour in girls but not boys. Nevertheless, the significant sex-specific effect of BPS on Language development (Bayley-III) was not observed for BPA. Although the structure of BPA and BPS are very similar, they might have some different toxicological mechanisms. For example, Castro et al. examined pre- and post-natal exposure to BPA and BPS and their effects in the brains of juvenile female rats. They reported that BPA and BPS decreased different 5α-reductase isozymes, an enzyme involved in neurosteroidogenesis.[67]

The average total BPS exposure was still significantly lower than for BPA (Table 5-4). However, the toxicological potency of BPS may be greater than that of BPA. For example, a study in embryonic zebrafish exposed to either BPA or BPS reported that low-dose BPS treatment resulted in a 240% increase in neurogenesis in the hypothalamus, higher than the effect caused by BPA treatment (180% increase).[39] It is also important to keep in mind that BPS is replacing BPA in certain applications and, in the USA, an increasing trend of BPS exposure has been shown from 2000 to 2014, while exposure to BPA has decreased.[38] Moreover, the maximum concentration (Cmax) of urinary BPS was 192 μg/g creatinine in the current study, three times as high as the Cmax for BPA (60 μg/g creatinine), thus further attention to the effects of BPS in humans is warranted.

A strength of this study was our ability to evaluate the modifying potential of methyl-donating nutrients on the observed associations between BPA and BPS exposure and neurodevelopment and behaviour. I was also able to adjust the final statistical models for a number of other maternal nutrients, and co-exposures to neurotoxic metals, as well as family income, maternal ethnicity, child's sex, gestational age of the child at birth, and child's age at assessment. Relationships between prenatal BPA exposure and outcomes were adjusted for BPS exposure. Thus, the observed effects of prenatal BPS exposure were independent from co-exposure to BPA.

The Bayley scales are objective and the most widely used measure of early development for young children.[43] However, Anderson et al. reviewed literature on the Bayley-III and concluded that the Bayley-III overestimates development measured by its Cognitive, Language, and Motor subscales.[68] This might decrease the sensitivity of Bayley-III and lead to a lower power to test associations between Cognitive, Language, Motor and prenatal BPA exposure. Different from Bayley-III, the CBCL is a parent-reported assessment of child behaviour and, thus, inherently subject to reporting bias or difficulty in inferring a child's internal state.[23]

There are limitations to this study. First, children's neurodevelopment and behaviour were only evaluated at 2 years of age, while it is known that neurodevelopment and behaviour continue to change

through childhood. For example, longitudinal analyses of children have revealed a decrease in aggressive behaviours from infancy to adolescence.[69,70] However, the children in this cohort have also been neurodevelopmentally assessed at ages 3 and 5 years, and developmental trajectories will be the subject of future data analyses. Another limitation of our study is the use of a single measurement of spot urine to categorize early pregnancy exposure to BPA and BPS. This limitation has been raised for epidemiological studies of BPA in the past [71] for two reasons. First, the biological half-life of BPA after oral exposures was less than 6 hours,[5,6] but I recently reported that the biological half-life is longer when exposure is by the dermal pathway, and the importance of the dermal pathway for total bisphenol exposure may be greater than previously assumed.[72] Second, the noise in BPA measurements resulting from its short biological half-life is expected to bias associations toward the null. Finally, another limitation is that APrON participants are mainly Caucasian, with high levels of education and family income, and the effects observed in the present cohort might not be generalizable to other racial, ethnic or social groups.

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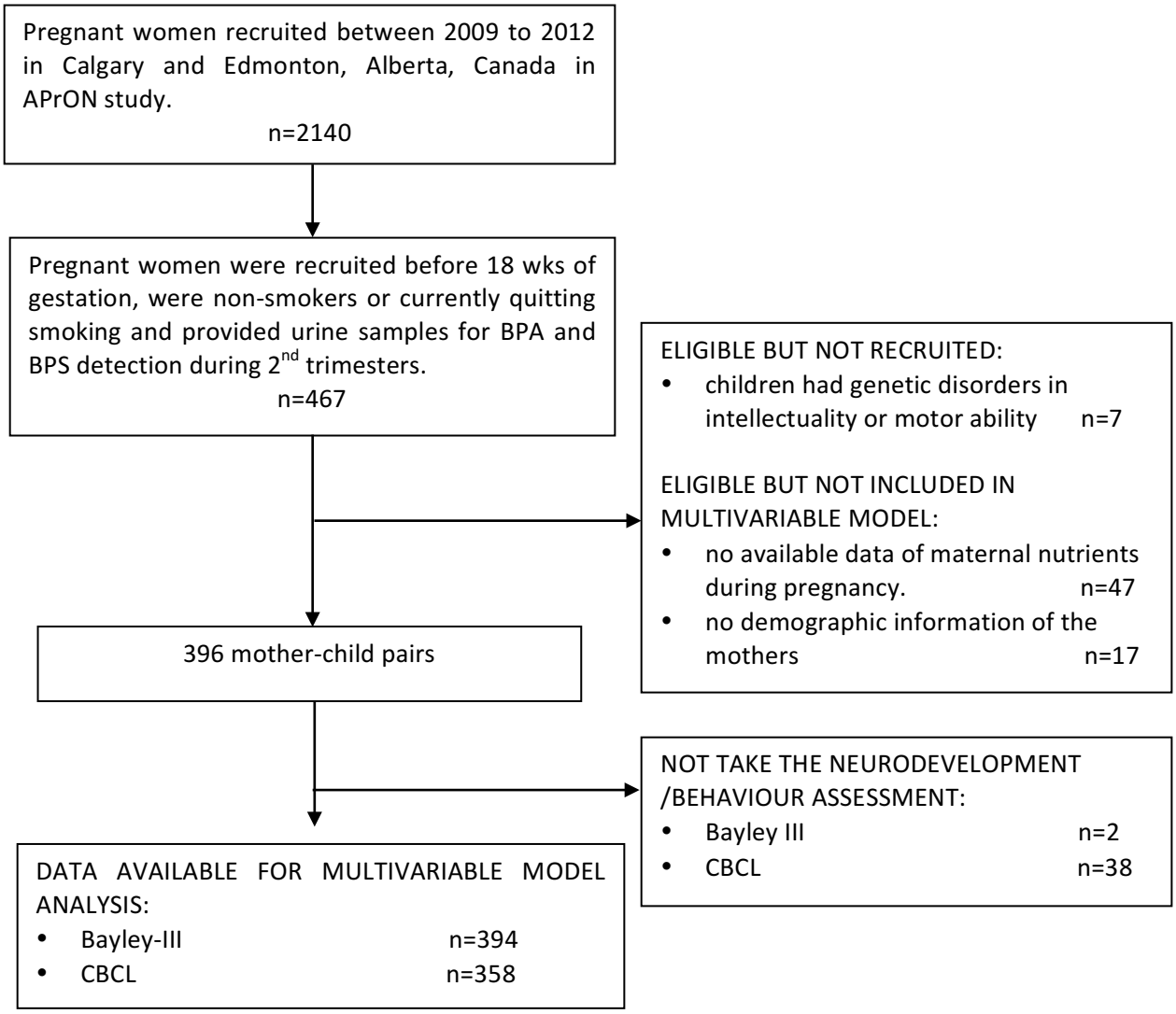

Figure 5-1 Summary of participant inclusion and exclusion criteria for this study in the APrON birth cohort.

Table 5-1 Maternal nutrients status and co-exposure of four heavy metals of our participants.

Nutrients	unit		Median (±SD)	Cutoff of deficiency/reference range	% of deficiency/<low reference level
Maternal nutrients					
Ferritin	ng/mL serum		38 (±36)	<12[74]	4.1% [b]
Hemoglobin	g/L whole blood		122 (±8.6)	<110[74]	1.5% [b]
Vitamin B12 (Plasma holoTC)	pmol/L		114 (±52)	<35[75]	0.6% [b]
Folate RBC	nmol/L		1305 (±543)[a]	<305[76]	0.5% [b]
Choline intake	mg/1000kcal		159 (±68)	No established reference intake as a percent of energy (adequate intake set at 450 mg/d)	
DHA	% of total serum phospholipid		2.3 (±1.1)	No established reference range	
AA	% of total serum phospholipid		6.6 (±1.9)	No established reference range	
Cu	ng/mL RBC		816 (±94)	616-929[77]	1.7% [c]
Zn	µg/mL RBC		9517 (±1412)	9.0-15[77]	37% [c]
Mn	ng/mL RBC		15 (±5.6)	9.4-38[77]	4.3% [c]
Se	ng/mL RBC		244 (±38)	227-365[77]	29% [c]
Heavy metals		Detection of frequency			n>high reference level
As	ng/mL RBC	74%	0.47 (±0.84)	0-4.9[77]	2
Cd	ng/mL RBC	76%	0.33 (±0.49)	0-6.3[77]	0
Hg	ng/mL RBC	90%	1.1 (±1.3)	0-6.3[77]	1
Pb	ng/mL RBC	99.6%	8.5 (±5.1)	0-37[77]	2

[a] Hematocrit corrected concentration
[b] % of deficiency
[c] <low-reference level

Table 5-2 Comparison of characteristics for participants included or excluded from the Bayley-III assessment.

	Participants included (n=394)	Participants excluded	*p*-value
Prenatal urinary BPA(ng/mL)	1.2 (1.1, 1.4)	1.1 (0.89, 1.5)	0.57
Prenatal urinary BPS(ng/mL)	0.16 (0.15, 0.18)	0.16 (0.12, 0.20)	0.85
Average age at delivery (years)	32 (±4.1)	32 (±4.0)	0.91
Gestational wks at sampling	17 (±1.7)	17 (±2.0)	0.50
Gestational wks at birth	39 (±1.8)	39 (±1.3)	0.32
Maternal education			0.20
Primary	4 (1.0%)	0	
High school	26 (6.6%)	1 (1.7%)	
College	67 (17%)	11 (19%)	
University	194 (49%)	27 (47%)	
Post-graduate	103 (26%)	18 (32%)	
Family income per year			0.56
<20,000	9 (2.3%)	1 (1.7%)	
20,000-39,999	16 (4.1%)	4 (7.0%)	
40,000-69,999	43 (11%)	7 (12%)	
70,000-99,999	88 (22%)	12 (21%)	
≥100,000	238 (60%)	33 (58%)	
Ethnicity			0.82
Caucasian	346 (88%)	49 (85%)	
Asian	28 (7.1%)	6 (10%)	
Latin American	11 (2.8%)	2 (3.5%)	
Others	9 (2.3%)	1 (1.7%)	
Children's sex			0.30
Female	200 (51%)	28 (44%)	
Male	194 (49%)	36 (56%)	

Values are geometric mean (95% CI), mean (±SD) or n (%). The *p*-value indicates differences between included and excluded participants using the t-test for continuous variables, chi-square for categorical variables and Cochran-Armitage trend test for ordinal variables.

Table 5-3 Comparison of characteristics for participants included or excluded in the CBCL assessment.

	Participants included (n=358)	Participants excluded	*p*-value
Prenatal urinary BPA(ng/mL)	1.2 (1.1, 1.4)	1.2 (0.99, 1.5)	0.91
Prenatal urinary BPS(ng/mL)	0.16 (0.14, 0.18)	0.16 (0.13, 0.20)	0.97
Average age at delivery (years)	32 (±4.0)	32 (±4.6)	0.99
Gestational wks at sampling	17 (±1.7)	17 (±1.9)	0.62
Gestational wks at birth	39 (±1.9)	39 (±1.2)	0.72
Maternal education			0.06
Primary	2 (0.6%)	2 (2.1%)	
High school	20 (5.6%)	7 (7.5%)	
College	57 (16%)	21 (23%)	
University	180 (50%)	41 (44%)	
Post-graduate	99 (28%)	22 (24%)	
Family income per year			0.28
<20,000	6 (1.7%)	4 (4.3%)	
20,000-39,999	16 (4.5%)	4 (4.3%)	
40,000-69,999	37 (10%)	13 (14%)	
70,000-99,999	83 (23%)	17 (18%)	
≥100,000	216 (60%)	55 (59%)	
Ethnicity			0.73
Caucasian	312 (87%)	83 (88%)	
Asian	28 (7.8%)	6 (6.4%)	
Latin American	9 (2.5%)	4 (4.2%)	
Others	9 (2.5%)	1 (1.1%)	
Children's sex			0.10
Female	187 (52%)	43 (43%)	
Male	171 (48%)	57 (57%)	

Values are GM (95% CI), mean (±SD) or n (%). The *p*-value indicates differences between included and excluded participants using the t-test for continuous variables, chi-square for categorical variables and Cochran-Armitage trend test for ordinal variables.

Table 5-4 Descriptive statistics of prenatal BPA and BPS concentrations among participants of the Bayley-III or CBCL.

Compounds	Detection frequency	Percentile					mean	Geometric mean
		5th	25th	50th	75th	95th		
Participants in Bayley-III assessment								
Prenatal BPA (μg/g)								
All children (n=394)	92%	<LOD	0.90	1.6	2.9	11	3.3	1.7
Girls (n=200)	93%	<LOD	0.90	1.6	2.8	13	3.2	1.7
Boys (n=194)	90%	<LOD	0.90	1.6	2.9	9.3	3.3	1.7
Prenatal BPS (μg/g)								
All children (n=394)	58%	<LOD	<LOD	0.20	0.40	1.6	1.0	0.23
Girls (n=200)	57%	<LOD	<LOD	0.18	0.37	1.6	1.6	0.22
Boys (n=194)	59%	<LOD	<LOD	0.23	0.44	1.8	0.49	0.24
Participants in CBCL assessment								
Prenatal BPA (μg/g)								
All children (n=358)	93%	<LOD	0.91	1.6	2.8	11	3.1	1.7
Girls (n=187)	94%	<LOD	0.97	1.7	2.8	13	3.4	1.8
Boys (n=171)	92%	<LOD	0.89	1.5	2.8	7.3	2.8	1.6
Prenatal BPS (μg/g)								
All children (n=358)	58%	<LOD	<LOD	0.19	0.40	1.5	1.0	0.22
Girls (n=187)	59%	<LOD	<LOD	0.18	0.37	1.6	1.5	0.22
Boys (n=171)	58%	<LOD	<LOD	0.22	0.44	1.5	0.46	0.23

Note: LODs are 0.32 ng/mL for BPA and 0.10 ng/mL for BPS.

Table 5-5 Distribution and sex-specific difference of Bayley-III composite scores and CBCL raw scores.

Developmental and behaviour assessment	Boys			Girls			*p*-value
	Score range	Mean (SD) /median	n(%) in borderline or clinical range[a]	Score range	Mean(SD) /median	n(%) in borderline or clinical range[a]	
Bayley-III composite scores (n=394)							
Cognitive	70-145	109(13)	0	85-145	113(14)	0	0.002
Language	68-141	108(14)	4 (2.1%)	62-165	113(15)	3 (1.5%)	<0.001
Motor	73-154	104(14)	6 (3.1%)	64-154	110(15)	6 (3.0%)	<0.001
Social emotional	70-135	105(13)	0	80-140	110(13)	0	<0.001
Adaptive behavior	64-139	101(12)	6 (3.1%)	67-140	106(12)	3 (1.5%)	<0.001
CBCL syndrome scores (n=358)							
Emotionally reactive	0-15	2.0(2.0)/1	10 (5.8%)	0-9	1.8(1.8)/1	10 (5.3%)	0.31
Anxious/depressed	0-9	1.3(1.6)/1	2 (1.2%)	0-9	1.4(1.7)/1	5 (2.7%)	0.42
Somatic complaints	0-12	1.6(2.0)/1	13 (7.6%)	0-20	1.4(1.8)/1	16 (8.6%)	0.98
Withdrawn	0-12	1.2(1.8)/1	7 (4.1%)	0-8	0.90(1.3)/1	4 (2.1%)	0.07
Sleep problems	0-14	3.0(3.0)/2	17 (9.9%)	0-12	3.1(2.7)/2	11 (5.9%)	0.97
Attention problems	0-7	2.2(1.7)/2	6 (3.5%)	0-6	1.8(1.6)/1	6 (3.2%)	0.01
Aggressive behaviour	0-24	8.9(5.6)/8	3 (1.8%)	0-33	7.6(5.6)/7	3 (1.6%)	0.04
CBCL composite scores (n=358)							
Internal problems	0-41	6.2(5.8)/5	10 (5.8%)	0-31	5.6(5.3)/4	6(3.2%)	0.43
External problems	0-30	11(6.8)/11	14 (8.2%)	0-41	9.5(7.0)/9	3(1.6%)	0.04

[a] Composite scores<80 was set as clinical range for Bayley-III subscales. T-score≥65 and ≥60 was set as borderline or clinical range for CBCL syndrome scores and CBCL composite scores, respectively.

Table 5-6 Associations between Bayley-III composite scores and prenatal BPA exposure (log10-transformed creatinine-adjusted concentration) and BPS exposure (upper quartile vs. other three quartiles) from multivariable linear regression models.

Bayley III Composite scores	Unadjusted model [a] (n=457) coefficients (95% CI)	Adjusted main effects model [b] (n=394) coefficients (95% CI)	Effect measure modification by sex [c] (n=394) *p*-value	Stratum-specific estimates, adjusted model with effect measure modification by sex [d]	
				Boys (n=194) coefficients (95% CI)	Girls (n=200) coefficients (95% CI)
BPA (log_{10}-transformed creatinine-adjusted concentration)					
Cognitive	2.8 (-0.22, 5.8)	0.20 (-2.8, 3.2)	0.30	†	†
Language	0.82 (-2.4, 4.0)	-0.25 (-3.5, 3.0)	0.92	†	†
Motor	0.46 (-2.8, 3.7)	-0.10 (-3.6, 3.4)	0.72	†	†
Social emotional	-0.022(-3.1, 3.1)	-0.61 (-3.8, 2.6)	0.005**	-5.1 (-9.6, -0.63)*	3.8 (-0.60, 8.3)
Adaptive behaviour	2.1 (-0.78, 5.0)	1.4 (-1.7, 4.4)	0.44	†	†
BPS (upper quartile vs. other three quartiles)					
Cognitive	-0.36 (-3.2, 2.5)	0.84 (-2.0, 3.7)	**0.070**	3.4 (-0.58, 7.4)	-1.9 (-6.0, 2.2)
Language	-0.94 (-4.0, 2.1)	0.36 (-2.8, 3.5)	0.044*	3.4 (-0.87, 7.8)	-2.9 (-7.4, 1.5)
Motor	-2.9 (-6.0, 0.11)	-2.0 (-5.3, 1.3)	**0.090**	0.75 (-3.8, 5.3)	-4.9 (-9.6, -0.20)*
Social emotional	-1.8 (-4.7, 1.1)	-0.94 (-4.0, 2.2)	0.86	†	†
Adaptive behaviour	-0.66 (-3.4, 2.1)	0.44 (-2.4, 3.3)	0.24	†	†

[a] All eligible participants who provided 2nd trimester maternal urine samples and completed Bayley III assessment were included in this test.

[b] For BPA estimates, the model adjusted sex, family income, ethnicity, child age at assessment (in month), maternal nutrients (vitamin B12, folate, copper, manganese, selenium). For BPS estimates, the model adjusted above confounders and BPA concentration (log_{10}-transformed creatinine-adjusted concentration).

[c] Modification effects were tested by adding interaction of BPA × sex / BPS × sex in the adjusted main effects model.

[d] These stratum-specific estimates (95% CI) by sex were calculated from adjusted regression model using n=394 observations; for BPA estimates, the model included the interaction of sex and BPA exposure; for BPS estimates, the model included the interaction of sex and BPS exposure.

† Not applicable: This model was not included because the effect measure modification was not statistically significant.

* *p*-value <0.05; ** *p*-value<0.01; *p*-value<0.10 is highlighted in bold.

Table 5-7 Modifying potential of maternal status of methyl donors (low/high) during pregnancy on the relationship between Bayley-III composite scores and prenatal BPA exposure (log10-transformed creatinine-adjusted concentration) and BPS (upper quartile vs. other three quartiles).

Bayley III Composite scores	Modification by methyl donor status [a] (n=394) *p*-value	Adjusted model with modification by both sex and methyl donor status [b] (n=394)		Stratum-specific estimates, adjusted model with modification by methyl donor status only [c]	
		Modification by sex *p*-value	Modification by methyl donor status *p*-value	Low methyl donor (n=338) coefficients (95% CI)	High methyl donor (n=56) coefficients (95% CI)
BPA (log_{10}-transformed creatinine-adjusted concentration)					
Cognitive [d]	0.56	†	†	†	†
Language [d]	0.83	†	†	†	†
Motor [d]	0.92	†	†	†	†
Social emotional	0.033*	0.019*	0.11	†	†
Adaptive behaviour	0.45	†	†	†	†
BPS (upper quartile vs. other three quartiles)					
Cognitive	**0.070**	0.13	0.10	†	†
Language	**0.079**	0.049*	0.12	†	†
Motor	0.32	**0.092**	0.42	†	†
Social emotional [e]	0.11	†	†	†	†
Adaptive behaviour	0.19	†	†	†	†

[a] Adjusted for sex, family income, ethnicity, child age at assessment (in month), maternal nutrients (methyl donor status, copper, manganese, selenium) and cadmium.

[b] Only test this model when modification of sex is significant.

[c] Only test this model when modification of sex is not significant while modification of methyl donor status was significant. These stratum-specific estimates (95% CI) by sex were calculated from adjusted regression model using n=394 observations; for BPA estimates, the model included the interaction of sex and BPA exposure; for BPS estimates, the model included the interaction of sex and BPS exposure.

[d] Interaction of BPS × sex (p<0.10) is included in the models in this column.

[e] Interaction of BPA × sex (p<0.10) is included in the models of this column.

† Not applicable: This model was not included because the effect measure modification was not statistically significant.

* p-value <0.05; ** p-value<0.01; p-value<0.10 is highlighted in bold.

Table 5-8 Associations between CBCL scores and prenatal BPA exposure (log10-transformed creatinine-adjusted concentration) and BPS exposure (upper quartile vs. other three quartiles) from multivariable linear regression models.

CBCL scores	Unadjusted model [a] (n=411) coefficients (95% CI)	Adjusted main effects model [b] (n=358) coefficients (95% CI)	Modification by sex [c] (n=358) *p*-value	Stratum-specific estimates, adjusted model with effect measure modification by sex [d]	
				Boys (n=171) coefficients (95% CI)	Girls (n=187) coefficients (95% CI)
BPA (log_{10}-transformed creatinine-adjusted concentration)					
CBCL syndrome scores					
Emotionally reactive	0.24(0.077,0.41)**	0.23 (0.04, 0.43)*	0.56	†	†
Anxious/depressed	0.23(0.035,0.42)*	0.16 (-0.07, 0.40)	0.44	†	†
Somatic complaints	-0.016(-0.21,018)	0.10 (-0.12, 0.32)	0.72	†	†
Withdrawn	-0.059(-0.29,0.18)	-0.17 (-0.46, 0.12)	0.80	†	†
Sleep problems	0.14(0.003,0.27)*	0.22 (0.07, 0.37)**	0.82	†	†
Attention problems	0.12(-0.048,0.28)	0.23 (0.04, 0.42)*	0.35	†	†
Aggressive behaviour	0.073(-0.009,0.15)	0.12 (0.03, 0.22)*	0.033*	0.02 (-0.11, 0.16)	0.22 (0.09, 0.35)**
CBCL composite scores					
Internalizing problems	0.13(0.030,0.22)*	0.11 (-0.005, 0.22)	0.27	†	†
Externalizing problems	0.076(0.003,0.15)*	0.14 (0.05, 0.22)**	0.033*	0.05 (-0.07, 0.17)	0.22 (0.11, 0.34)**
BPS (upper quartile vs. other three quartiles)					
CBCL syndrome scores					
Emotionally reactive	0.086(-0.073,0.24)	0.06 (-0.12, 0.23)	**0.083**	-0.10 (-0.35, 0.15)	0.21 (-0.03, 0.46)
Anxious/depressed	0.25(0.068,0.43)**	0.19 (-0.01, 0.39)	0.67	†	†
Somatic complaints	0.063(-0.11,0.24)	0.003(-0.20, 0.20)	0.97	†	†
Withdrawn	-0.061(-0.28,0.16)	-0.07 (-0.31, 0.18)	0.98	†	†
Sleep problems	0.10(-0.02,0.23)	0.14 (0.00, 0.27)*	0.79	†	†
Attention problems	0.060(-0.095,0.22)	0.03 (-0.14, 0.21)	0.81	†	†
Aggressive behaviour	0.038(-0.039,0.12)	0.02 (-0.06, 0.11)	0.007**	-0.09 (-0.21, 0.03)	0.15 (0.02, 0.27)*
CBCL composite scores					
Internalizing problems	0.11(0.016,0.20)	0.02 (-0.08, 0.12)	0.47	†	†
Externalizing problems	0.032(-0.037,0.10)	-0.02 (-0.09, 0.06)	0.29	†	†

[a] All eligible participants who provided 2nd trimester maternal urine samples and completed CBCL assessment were included in this test.

[b] For BPA estimates, the model adjusted sex, family income, ethnicity, gestational weeks at birth, maternal nutrients (ferritin, vitamin B12, folate, choline, AA, copper, zinc, manganese and selenium) and metal neurotoxicants (cadmium, total mercury and lead). For BPS estimates, the model adjusted above confounders and BPA concentration (log_{10}-transformed creatinine-adjusted concentration).

[c] Modification effects were tested by adding interaction of BPA × sex / BPS × sex in the adjusted main effects model.

[d] These stratum-specific estimates (95% CI) by sex were calculated from adjusted regression model using n=358 observations; for BPA estimates, the model included the interaction of sex and BPA exposure; for BPS estimates, the model included the interaction of sex and BPS exposure.

† Not applicable: This model was not included because the effect measure modification was not statistically significant.

* *p*-value <0.05; ** *p*-value<0.01; *p*-value<0.10 is highlighted in bold.

Table 5-9 Modifying potential of maternal status of methyl donors (low/high) during pregnancy on the relationship between CBCL scores and prenatal BPA exposure (log10-transformed creatinine-adjusted concentration).

CBCL	Modification by methyl donor status [a] (n=358) *p*-value	Adjusted model with modification by both sex and methyl donor status [b] (n=358)		interaction of BPA × sex × methyl donors [c] (n=358) *p*-value	Stratum-specific estimates, adjusted model with modification by methyl donor status [d]	
		Modification by sex *p*-value	Modification by methyl donor status *p*-value		Low methyl donor (n=303) coefficients (95% CI)	High methyl donor (n=55) coefficients (95% CI)
CBCL syndrome scores						
Emotionally reactive [e]	0.83	†	†	†	†	†
Anxious/depressed	**0.085**	†	†	†	0.21 (-0.03, 0.46)	-0.36 (-0.98, 0.25)
Somatic complaints	0.66	†	†	†	†	†
Withdrawn	0.79	†	†	†	†	†
Sleep problems	**0.093**	†	†	†	0.27 (0.11, 0.43)**	-0.09 (-0.49, 0.30)
Attention problems	0.13	†	†	†	†	†
Aggressive behaviour [e]	0.049*	0.019*	0.011*	0.11	†	†
CBCL composite scores						
Internalizing problems	0.63	†	†	†	†	†
Externalizing problems	0.019*	0.011*	0.005**	0.38	†	†

[a] Adjusting for sex, family income, ethnicity, gestational weeks at birth, maternal nutrients (methyl donor status, ferritin, AA, copper, zinc, manganese and selenium) and metal neurotoxicants (cadmium, total mercury and lead).

[b] Only test this model when interaction of sex is not significant while interaction of methyl donor status was significant.

[c] All first order interactions are included in the model for testing the second order interaction.

[d] Only test this model when modification of sex is not significant while modification of methyl donor status was significant. These stratum-specific estimates (95% CI) by sex were calculated from adjusted regression model using n=358 observations; for BPA estimates, the model included the interaction of sex and BPA exposure; for BPS estimates, the model included the interaction of sex and BPS exposure.

[e] Interaction of BPS × sex (p<0.10) is included in the models of this column.

† Not applicable: This model was not included because the effect measure modification was not statistically significant.

* p-value <0.05; ** p-value<0.01; p-value<0.10 is highlighted in bold.

Table 5-10 Modifying potential of maternal status of methyl donors (low/high) during pregnancy on the relationship between CBCL scores and prenatal BPS exposure (upper quartile vs. other three quartiles).

CBCL	Modification by methyl donor status [a] (n=358) *p*-value	Adjusted model with modification by both sex and methyl donor status [b] (n=358)		interaction of BPS × sex × methyl donors [a] (n=358) *p*-value	Stratum-specific estimates, adjusted model with modification by methyl donor status [c]	
		Modification by sex *p*-value	Modification by methyl donor status *p*-value		Low methyl donor (n=303) coefficients (95% CI)	High methyl donor (n=55) coefficients (95% CI)
CBCL syndrome scores						
Emotionally reactive	**0.056**	**0.071**	**0.095**	0.43	†	†
Anxious/depressed	0.46	†	†	†	†	†
Somatic complaints	0.61	†	†	†	†	†
Withdrawn	0.25	†	†	†	†	†
Sleep problems	0.49	†	†	†	†	†
Attention problems	0.32	†	†	†	†	†
Aggressive behaviour [d]	0.57	0.026*	0.62	†	†	†
CBCL composite scores						
Internalizing problems	**0.099**	†	†	†	-0.01(-0.12, 0.10)	0.23(-0.03, 0.48)
Externalizing problems [d]	0.21	†	†	†	†	†

[a] These models adjust for sex, family income, ethnicity, gestational weeks at birth, maternal nutrients (methyl donor status, ferritin, AA, copper, zinc, manganese and selenium) and metal neurotoxicants (cadmium, total mercury and lead).

[b] Only test this model when modification of sex is moderate significant (p<0.10).

[C] Only test this model when modification of sex is not significant while modification of methyl donor status was significant.

[d] Interaction of BPA × sex and BPA × methyl donors (p<0.10) are included in the models of this column.

† Not applicable: This model was not included because the effect measure modification was not statistically significant.

p-value<0.10 is highlighted in bold.

Table 5-11 Modified associations between BPA exposure (log10-transformed creatinine-adjusted concentration) and CBCL scores when significant (p<0.05) modifications of methyl donor status and sex are observe.

CBCL scores	Stratum-specific estimates, adjusted model with modification by both sex and methyl donor status [a]			
	Boys (n=171)		Girls (n=187)	
	Low methyl donor (n= 149)	High methyl donor (n=22)	Low methyl donor (n=154)	High methyl donor (n=33)
Aggressive behaviour [b]	0.053 (-0.083,0.19)	-0.29 (-0.57, -0.01)*	0.27 (0.14, 0.41)**	-0.073 (-0.32, 0.18)
Externalizing problems	0.069 (-0.052,0.19)	-0.27 (-0.52,-0.018)*	0.28 (0.16,0.40)*	-0.057 (-0.28, 0.17)

[a] These models adjust for sex, family income, ethnicity, gestational weeks at birth, maternal nutrients (methyl donor status, ferritin, AA, copper, zinc, manganese and selenium) and metal neurotoxicants (cadmium, total mercury and lead).

[b] Interaction of BPS × sex (p<0.05) is included in the models of this column.

* p-value <0.05; ** p-value<0.01

6 Conclusions and Synthesis

6.1 Summary of previous knowledge

BPA and its alternatives, BPS and BPF, are endocrine disruptors [1] that are widely used in consumable products including in food cans, for water bottles, toys, dental materials and on thermal paper receipts. [2–5] National surveys in the USA and Canada have shown that BPA is detectable in >90% of urine samples, [6,7] while BPS and BPF are also now frequently detected in urine of Americans. [8] Although the urinary concentrations of BPF and BPS were generally lower than BPA, the 95th percentile of BPF was comparable or higher than that of BPA, and exposure to BPS was increasing between 2000 and 2014.[8]

Diet, especially canned food, has been regarded as the main exposure source of BPA,[9–13] while less is known regarding exposure sources of BPA alternatives. BPF was the most predominant BPA alternative detected in various food items collected from retail grocery stores in the USA and China.[9,14] BPS was also detected in foodstuff in these studies, but its concentrations were generally 1-2 orders of magnitude lower than BPA and BPF. Another known exposure route of these analogues is dermal exposure via thermal paper contact, and for BPA it has been estimated that 10% of total exposure may be through the skin for some age groups. [15] A high concentration of BPS was also detected in paper products including retail receipts, airplane luggage boarding passes, tickets and paper currencies.[16] An increase of urinary BPS concentration was observed in cashiers after handling receipts containing BPS during work shift.[17]

After dietary exposure, free BPA, the toxic form of BPA, is completely metabolized to non-toxic metabolites and quickly excreted from urine.[18,19] The half-life of total BPA following oral exposure was less than 6 hours, and the percentage of free BPA in serum was less than 1% of total BPA in human pharmacokinetic studies.[18,19] BPA or BPA-alternative exposure through skin would bypass efficient first-pass metabolism in the intestine and liver, and might lead to a higher proportion of free BPA (the toxic form) in systemic circulation. Thus, dermal exposure could lead to different, and perhaps greater toxicological effects than dietary exposure.[15]

Pregnancy is a critical window of exposure due to developmental processes in the fetus. It is important to evaluate the exposure to BPA and its alternatives during pregnancy, their placental transfer, and the adverse effects this may have on the developing child. Total BPA has already been detected in maternal serum and cord serum from different countries,[20–23] suggesting that maternal exposure to BPA during pregnancy can lead to *in utero* exposure of the fetus. Low placental transfer efficiency and

deconjugation of BPA metabolites in the fetus were observed in an animal model,[24] however, biomonitoring of BPA metabolites in human maternal and fetal serum remains limited, and even less is known for placental transfer of BPA alternatives.

In animal studies, perinatal exposure to BPA caused developmental defects [25] and alterations in brain and behaviours [26] with variance across age and sex. For example, developmental exposure to BPA increased anxious behaviour of female mice in a dose-dependent fashion (male mice were not tested).[27] In contrast, perinatal exposure of BPA within the range of human exposure reduced the anxiety and motivation to explore in male rats, and depressed the motor activity and motivation to explore in female rats.[28] The neurodevelopmental effects of perinatal and childhood exposure to BPA were evaluated in epidemiological studies, but the observed sex-specific effects were not consistent. For example, Harley et al. observed that prenatal BPA exposure levels were associated with increased internalizing problems (i.e. anxiety and depression) in boys but not in girls (n=292) at age of 7 years.[29] Using the same behaviour assessment method, Braun et al. also reported that gestational BPA exposure was positively associated with anxious and depressed behaviour at 3 years of age (n=244), but the associations were stronger among girls than boys.[30] The difference in study designs including assessment age of the children, urine collection time for evaluating BPA exposure and un-controlled confounders may account for these inconsistent results.

Child's sex, family income, maternal education, maternal age, gestational age, smoking status and ethnicity were commonly adjusted as confounders for evaluating the effects of BPA on neurodevelopment of children.[31,32] However, no epidemiological study of BPA has previously considered maternal nutrient status during pregnancy, nor co-exposure to other classic neurotoxicants. Maternal nutritional status during pregnancy has been shown to affect fetal growth and has the potential to impact on future health conditions.[33,34] For example, children with chronic iron deficiency in infancy had higher developmental and behavioural risk even after 10 years of iron treatment.[35] Besides maternal nutrients, neurotoxicants, which might have similar exposure sources as BPA should be considered as confounders for neurodevelopment of children. For example, high levels of heavy metals have been detected in canned fish,[36] which is also regarded as an important exposure source of BPA.[12,13]

In order to fill many knowledge gaps, I investigated BPA metabolites and BPA-alternatives in paired maternal and cord sera (Chapter 2), and evaluated the pharmacokinetics of dermal exposure in humans using isotope-labeled BPA (Chapter 3). In Chapters 4 and 5, which utilized the APrON cohort

study, I had high statistical power to investigate dietary exposure sources of bisphenols in pregnant women, as well as the subsequent adverse effects on neurodevelopment of children at age 2.

6.2 Advances in knowledge

6.2.1 *in utero* exposure of BPA metabolites and BPS

To evaluate the *in utero* exposure to major BPA metabolites and BPA alternatives, I developed a method for detecting BPA-glucuronide, BPA-sulfate, BPA-bissulfate, BPS, BPB and BPAF in human serum, and analyzed 61 paires of maternal and cord serum from China. Among BPA alternatives, only total BPS was detectable, and only in 4 maternal sera (<0.03-0.07 ng/mL) and 7 cord sera (<0.03-0.12 ng/mL), indicating relatively low exposure to BPS compared to BPA, yet providing the first evidence that BPS crosses the human placenta. No free BPS was detected in any serum sample, suggesting that BPS was predominantly present in its conjugated forms. Statistics were not possible on this small data subset, but among the 11 samples with detectable total BPS, 4 of these were for paired maternal and cord sera (i.e. 2 paired samples): individual maternal concentrations were 0.07 and 0.03 ng/mL, while those in paired cord serum were 0.08 and 0.04 ng/mL, respectively, suggesting that higher fetal BPS levels were associated with higher maternal concentrations, and that placental transfer was efficient.

BPA-glucuronide and BPA-sulfate were detectable in cord and maternal serum with detection frequencies in the range of 36% to 66% (>0.01 ng/mL). BPA-bissulfate was monitored but was not detectable (<0.20 ng/mL) in the present study. In both maternal and cord sera, BPA-sulfate was the major metabolite detected (GM: 0.06 ng/mL and 0.08 ng/mL, respectively), significantly higher than BPA-glucuronide (GM: 0.02 ng/mL and 0.04 ng/mL, respectively) (p<0.01). Furthermore, the proportion of BPA-sulfate increased with total BPA. These results for pregnant women are markedly different from the pharmacokinetic after oral exposure studies, whereby BPA-glucuronide is the major BPA metabolite. One explanation for the dominant BPA-sulfate concentrations in the present study is unique biotransformation pathways in the human fetoplacental compartment, which lead to higher proportions of BPA-sulfate in cord blood, and moreover may have influenced the maternal serum metabolite profile through placental transfer back to maternal circulation. Total BPA metabolites in cord serum were significantly higher than in maternal serum (p<0.05), suggesting that these metabolites may be formed in the fetus or are cleared more slowly from the fetoplacental compartment.

These are the first human data for BPA metabolites and BPA alternatives in paired maternal and cord serum. The results suggest that the human fetus and pregnant mother have a unique exposure to BPA metabolites that would be missed if only 'total BPA' analysis is used.

6.2.2 Exposure and dietary sources of BPA and BPA alternatives

Dietary intake is regarded as the main source of BPA exposure, but few studies have evaluated the importance of diet for exposure to BPA alternatives. In Chapter 4, free and total BPA and BPA alternatives were monitored in 467 second trimester urine samples and 455 paired samples collected three months postpartum. Free BPA, free BPS and free BPF were only detectable in 3, 9 and 1 out of the 467 urine samples collected in the second trimester. Cmax of free BPS and BPF were detected in the same samples containing Cmax of total BPS and BPF, respectively, in proportions of <1% out of total BPS or BPF. The pharmacokinetics of BPA alternatives have not been evaluated in any controlled *in vivo* study in humans, but the low detection frequency and small proportion of the free forms of BPA and BPA alternatives here support the notion that these phenols can be efficiently metabolized in humans.

The GM's of total urinary concentration of BPA in the second trimester (1.2 ng/mL) and 3 months postpartum (0.95 ng/mL) were 5-7 times higher than the associated values for BPS (0.16 ng/mL, 0.17 ng/mL). However, Cmax of total BPA pre- and post-partum (44 ng/mL, 55 ng/mL) was much lower than Cmax for total BPS (243 ng/mL, 72 ng/mL) or for total BPF (390 ng/mL,115 ng/mL), and the 95th percentile of total BPF was comparable with BPA. In addition, the maximum estimated 24 hr intake of BPS (14 nmol/kg BW/day), and the top 5 estimated 24 hr intakes of BPF (21-30 nmol/kg BW/day) were in the same range or exceeded the TDI of BPA (18 nmol/kg BW/day, EFSA[37]). The high exposure to BPS and BPF in a minority of pregnant women from the background Canadian population highlights the need to monitor exposure sources and levels of these BPA alternatives.

The 24-hr food recall data showed that the consumption of canned foods, especially canned meat, was associated with higher urinary total BPA. In contrast for BPS, consumption of canned food, or of any meat and meat products, was not associated with urinary levels. Due to the low detection frequency, statistical methods could not be used to evaluate the dietary sources of BPF, but the percentage of canned food consumption in participants who had detectable urinary BPF (40%; n=35) was comparable with those who did not have detectable urinary BPF (44%; n=334). The participant who had Cmax of urinary BPF also did not consume any canned food in the 24 hrs preceding sample collection. In addition, BPS was the main bisphenol detected in 9 of 20 randomly collected thermal receipts from stores in Edmonton, Alberta, Canada. Taken together, these results lead us to a hypothesis that dermal exposure

from thermal paper could be a major exposure source of BPS in the general population. More studies are needed to evaluate the non-dietary sources of BPA alternatives.

6.2.3 Different pharmacokinetics of BPA following dermal and dietary exposure

Dermal exposure to BPA from thermal paper receipts is already recognized as the most important non-dietary exposure pathway, and is estimated to contribute 7-15% of total BPA exposure in all population groups above 3 years of age.[38] However, this estimate is based on uncertain in vitro experiments,[38] thus the true pharmacokinetics of BPA following in vivo dermal exposure are not well characterized in people. To compare and contrast the pharmacokinetics of dermal and dietary BPA exposure, six male participants first handled simulated receipts containing relevant levels of BPA (isotope-labeled BPA-d_{16}) for 5 min, followed by hand-washing 2 hrs later. Urine (0-48 hrs) and serum (0-7.5 hrs) were monitored for free and total BPA-d_{16}. Then, one week later, participants returned for a dietary administration with monitoring as above. One participant repeated the dermal administration with extended monitoring of urine (9 days) and serum (2 days). Large differences in pharmacokinetics were observed between the dermal and dietary exposure pathways. From the dietary pathway, total BPA-d_{16} reached peak concentrations in serum within 4 hrs and >96% of administrated BPA-d_{16} was excreted in urine within 12 hrs. By contrast, total BPA-d_{16} was not even detectable in serum within 7.5 hrs post dermal exposure, but was indeed detectable by 22 hrs and was still present after 51 hrs at a comparable concentration (0.29 and 0.30 ng/mL total BPA-d_{16}). Cumulative excretion increased linearly for the 2 days of sampling after dermal exposure, and increased linearly over 5 days in the follow up dermal exposure. Among the 4 participants with detectable data from both dermal and dietary exposures, I reported a statistically significant difference in the pharmacokinetics based on the T_{max}, and slopes of the cumulative excretion curves between the two routes of exposure (p<0.05). Despite the small number of participants, both tests had high power (>0.95, based on 5% type I error) to detect differences. These results clearly demonstrated a prolonged exposure to BPA following single dermal contact events. Moreover, an interesting diurnal urinary concentration pattern was observed over the first 5 days, whereby concentrations of BPA-d_{16} were highest in the first morning's urine and decreased at night. All these data may be useful in design and interpretation of future exposure assessment studies of BPA.

Free BPA-d_{16}, the toxic form of BPA-d_{16}, was detected in the participant's serum collected 1 and 2 days after dermal exposure. The proportion free BPA-d_{16} was 62% and 84% of total BPA-d_{16} in these two serum samples. In contrast, no free BPA-d_{16} was ever detectable in serum after the dietary exposures.

Based on the serum LOD of BPA-d_{16} in the present study, the proportion of free BPA-d_{16} was estimated to be < 18% in the serum sample with the highest total BPA-d_{16} after dietary exposure. Proportions of free BPA-d_{16} in urine following dermal exposure (GM: 2.2% of total BPA-d_{16}) were also much higher than after dietary exposure (GM: 0.6%). These results suggest a lower metabolism efficiency of BPA following dermal uptake, and raise concerns for elevated health risks following dermal exposure. It is a hypothesis that dermal exposure to BPA may partly explain why adverse associations are routinely noted in environmental epidemiology studies of BPA,[39] despite that exposure is usually below the TDI.

6.2.4 Effects of prenatal exposure to BPA and BPS on neurodevelopment of children

Experimental evidence and epidemiological studies suggest that BPA is a neurotoxicant with sex-specific effects, but previous epidemiological studies did not consider the confounding effects of maternal nutrient status or co-exposure to classic neurotoxicants, nor the interaction between BPA exposure and intake of methyl donors. BPS, a common alternative to BPA, is now frequently detected in human biofluids, yet epidemiological study on BPS is limited. To examine sex-specific effects of maternal BPA and BPS exposure on subsequent child neurodevelopment at two years of age, total BPA and BPS concentrations were analyzed in spot urine samples from pregnant women in the second trimester. Exposure of classic neurotoxic metals and maternal nutrients status, including methyl donors (folate, vitamin B12 and choline) were evaluated using blood samples or dietary data collected during pregnancy. At age 2, child neurodevelopment was assessed by the Bayley-III scales (n=394), and behavioural syndromes were evaluated using the CBCL (n=358). After adjusting potential confounders including maternal nutrients status and co-exposure to neurotoxic metals, BPA exposure was negatively associated with Social Emotional scores for boys only (β=-5.1; 95% CI: -9.6, -0.63), while higher prenatal BPA exposure was positively associated with 3 of 7 CBCL syndrome scales (emotionally reactive, sleep problems, attention problems) in all children and, among girls only, with aggressive behaviour and externalizing problems. These results indicate that prenatal exposure of BPA is negatively associated with indices of child development, and positively associated with behavioural problems at 2 years of age, with sex-specific effects.

To our knowledge, I have evaluated for the first time the effects of prenatal BPS exposure on neurodevelopment of children. Although the average exposure of BPS was approximately 7 times lower than for BPA, higher BPS exposure was associated with lower Motor scores (worse performance) for girls only (β=-4.9; 95% CI: -9.6, -0.20), more reported sleep problems in all children and more aggressive behaviours in girls, even after adjusting for BPA exposure, maternal nutrient status and co-exposure to

neurotoxic metals. Consistent with the sex-specific effects of BPA, a significant BPS × sex interaction was observed on aggressive behaviour, whereby higher prenatal bisphenol exposure was significantly associated with more aggressive behaviour in girls, but not boys. These results suggest that BPS may not be any safer than BPA.

A strength of this study was the ability to evaluate the modifying potential of methyl donor intake during pregnancy on the adverse effects of BPA exposure on neurodevelopment of children. Higher maternal status of methyl donors seemed to counteract the adverse effects of BPA exposure on CBCL scores of Aggressive bahaviour and Externalizing problems. Although the interaction of BPA × methyl donors was not significantly modified by child sex, the modifying effects of methyl donor status and child's sex were significant on these two CBCL scores, while the three-way-interaction of BPA × sex × methyl donors was not significant. This is the first epidemiological study to evaluate the modifying effects of methyl donors on the relationship between prenatal BPA exposure and neurodevelopment. The modifying effects of methyl donors on the effects of BPA were previously observed in animal studies, whereby maternal supplementation of methyl donors, such as folic acid, modified the effects of prenatal BPA exposure on DNA hypomethylation and counteracted subsequent changes in coat color of viable yellow agouti mice.[40] Supplementation of methyl donors also modified the adverse effects of BPA exposure on intestinal digestion and absorption function in pigs, which might be related to DNA methylation.[41] More studies are needed to evaluate the combined effects of prenatal BPA exposure and methyl donors on genome-wide epigenomic alterations and neurodevelopment of children.

6.3 Conclusions of thesis

Based on the results from Chapter 2 to Chapter 5, I conclude that: 1) the pregnant woman and fetus have a unique exposure to BPA metabolites that is unlike that in non-pregnant humans; 2) Dermal exposure to BPA leads to prolonged exposure and lower metabolism efficiency compared to the oral route; 3) canned food is a dominant exposure source for BPA, but the same is not true for BPS; 5) both BPA and BPS have sex-specific effects on neurodevelopment and behaviors of children, while maternal methyl donor status/intake seems to counteract some of the effects of BPA.

6.4 Future research

Human biomonitoring of BPA exposure has generally relied on detection of total BPA in urine. However, based on the result in Chapter 3, total BPA in urine may not be an adequate estimate of total or free BPA in circulation resulting from a mixture of oral and dermal exposure, and may not capture the distinct exposure to BPA-sulfate that occurs for pregnant women in late pregnancy. Free BPA in human serum could be a good biomarker of non-dietary BPA exposure, and may be more relevant to risk estimation than urinary total BPA. However, avoiding contamination from free BPA during sampling of blood is difficult to achieve.[42,43] Additionally, the total and free BPA-d_{16} in serum following the dermal exposure at an environmental relevant concentration (Chapter 3) was very low (0.02 – 0.03 ng/mL), and would not be detectable by today's routine methods with LODs in the range of 0.05-0.35 ng/mL.[44,45] Before biomonitoring of BPA in blood will be widely acceptable, future studies will need to improve method sensitivity through quality control and alternate sample preparation strategies or instrumental methods.

Due to the restrictions of BPA in certain commercial products such as baby bottles, BPA alternatives such as BPS and BPF have been used in a number of consumer products.[5] The study in Chapter 4 showed that BPS and BPF were detected in 59% and 9% of urine samples (>0.10 BPS ng/mL, >1.0 BPF ng/mL) from pregnant women (n=467) in Canada. Although the GM of urinary BPS in participated women was 5-7 times lower than total urinary BPA in second trimester and postpartum, the Cmax of total BPS (243 ng/mL, 72 ng/mL) and BPF (390 ng/mL,115 ng/mL) was much higher than that of total BPA (44 ng/mL, 55 ng/mL), and 95th percentile of BPF was comparable with BPA. In addition, a study in the USA observed an increase of BPS concentrations in convenience urine samples from USA adults between 2000 and 2014, while urinary concentration of BPA decreased during this period.[8] Furthermore, higher exposure of BPS during pregnancy was found to be significantly associated with the neurodevelopment and behaviour syndromes of children in Chapter 5 of this thesis, indicating that BPS might not be any safer than BPA. More studies are needed to monitor the exposure sources and levels of these BPA alternatives, and to evaluate the health risks of their exposure in humans.

Different from BPA, the level of urinary BPS was not associated with consumption of canned food or of any meat and meat products (Chapter 4), indicating higher contribution from non-dietary exposure sources for total BPS exposure compared to BPA. I randomly collected 20 thermal receipts from local stores in Edmonton, Alberta, Canada and found that the main bisphenol on 9 of 20 receipts was BPS, not BPA. Thus, dermal exposure from thermal paper could be an important exposure pathway of BPS in general population. Given the similar structure of BPS compared to BPA, dermal exposure to BPS is also

hypothesized to lead to a prolonged exposure, and a higher proportion of free BPS in circulation. However, the pharmacokinetics of BPS during a dermal exposure have not been studied in vivo, nor in any in vitro model. An in vitro study of percutaneous BPS absorption or an in vivo human study of the pharmacokinetics of dermal exposure to BPS, compared to BPA in the same study, would be very helpful to accurately assess the health risk of using BPS as an alternative for BPA in various applications, including thermal paper. A potential barrier to controlled studies of BPS exposure in humans are research ethical considerations of purposely exposing people to a compound with no defined TDI.

Higher status/intake of methyl donors during pregnancy was found to counteract the adverse effects of prenatal BPA exposure on both neurodevelopment (Bayley-III scores) and behavioural syndromes (CBCL scores) of children (Chapter 5). This suggested that the effects of BPA on neurodevelopment of children are mediated through epigenetics, and can be mitigated by supplementation of methyl donors during pregnancy, as has been demonstrated in animals.[40,41] However, it is not clear how methyl donors modified the effects of BPA on neurodevelopment, how genomic DNA methylation is involved in this mechanism, and whether postnatal supplementation of methyl donors could also counteract the adverse effects of prenatal exposure to BPA. More epidemiological studies are needed to confirm the results I observed in Chapter 5. Animal or cell studies evaluating the combined effects of BPA exposure and methyl donors on genome-wide epigenomic alterations in the brain might be helpful to understand the molecular mechanisms that underlie the effects of BPA on neurodevelopment, as well as the modifying effects of methyl donors.

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