厚生労働科学研究費補助金
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前向きコーホート研究に基づく先天異常、免疫アレルギーおよび小児発達障害のリスク評価と環境化学物質に対する遺伝的感受性の解明

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I　はじめに

PCBs・ダイオキシン類や有機フッ素化合物、有機塩素系農薬などは、環境での蓄積性や残留性が強いことから、いわゆるPOPs（Persistent Organic Chemicals）として、世界的に生産や使用が制限されていますが、半減期が長いため、私たちの体内から容易にはなくなりません。さらに最近使用が増えてきているプラスチック可塑剤などとして使われているフタル酸エステル類やビスフェノールAなどは、半減期は短いのですが広範囲に使用されているため、多くの人々の血液から検出されます。このような化学物質の人への影響は、大人よりも小児、あるいは胎児期に大きいことが懸念されています。たとえば尿道下裂・停留精巣をはじめとする先天異常は、各国で増加が示唆されていますが、その原因が器官形成期のアンドロゲンの作用に依存しますので、いわゆる環境ホルモン作用を示すといわれるPCBs・ダイオキシン類などとの関係を調べる必要があります。しかし、世界的にも環境化学物質の次世代への直接的な影響と因果関係は、未だ解明されるには至っておりません。さらに、ADHDなど小児の軽度発達障害、あるいはアレルギーは、最近各国で増加しているとの報告が発表されていますが、わが国では地域の一定集団を対象に環境リスク要因を評価することが全くなされていませんでした。

そこで、私たちは2002年から2つの前向き研究を立ち上げて研究を行っております。本報告書は、直近3年間分の研究成果報告になります。札幌市の1産院で説明し同意を得た妊婦様514人の母体血とお子様の臍帯血について、PCBs・ダイオキシン類、水酸化PCB類、有機フッ素化合物（PFOS・PFOA）、農薬およびビスフェノールAの測定を行い、種々のアウトカムとの関係を調べています。また、北海道全域の北海道（大規模）コーホートでは、母子ペアで参加者が2万組を超えました。妊娠初期に同意を得た妊婦様全員の葉酸、コチニン濃度を測定し、母体血の有機フッ素化合物もより詳しくPFDA・PFNA・PFHxS・PFUnDAなど11種類の測定を行い、出生時体重やアレルギーへの影響を研究しています。今後は、先天異常や疾病との関係について解析を進めます。いずれのコーホートも、次世代影響を評価するために思春期まで追跡させていただくよう、参加者の皆様にお願いしています。

一方、最近のゲノムおよびエピゲノム研究の進歩から、化学物質の影響の強さは、曝露された個体の異物（薬物）代謝酵素類の遺伝子多型や疾病感受性遺伝子、あるいはメチル化等によっても修飾されることが考えられます。このような研究は、同じ曝露濃度でも遺伝的ハイリスク群である場合、より予防的な対応を進める必要があること、また環境の影響が遺伝子を介さず次世代に及ぶという意味で重要と思われます。

本研究は、産婦人科医療機関など臨床家と環境疫学専門家の協力で進めております。このような地域をベースに胎児期から立ち上げ、環境リスク評価を行っている研究は、最近少しずつ増えていますが、本研究は、世界的にもその先駆けと申し上げられます。今後、数多くの成果が出ることが期待されますので、引き続き皆様のご協力をお願いいたします。

最後に多くの皆様のご尽力により研究を継続することができ、過去3年間の報告書をこのようにまとめることができましたことに対して、衷心より御礼申し上げます。

平成26年3月

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Cohort Profile of the Hokkaido Birth Cohort Studies updated 2013

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研究要旨

「環境と子どもの健康に関する北海道スタディ」は前向き出生コーホート；札幌（小規模）コーホート（N=514）および北海道全域の37協力医療機関（図1）に協力を得た北海道（大規模）コーホート（N=20,929）からなる。2002年と2003年に開始した2つの研究の主な目的は、(1) 胎児期の環境化学物質曝露が先天異常や発育遅延など、児の出生に与える負の影響の解明、(2) 児の発育およびアレルギーや発達障害などを追跡し、環境化学物質がこれらのアウトカムに及ぼす影響の解明、(3) 環境化学物質曝露に対し遺伝的に脆弱なSNPsの特定、(4) 受動喫煙や妊娠中の葉酸欠乏といった日常的な環境要因の複合影響についての解明と、(5) エピゲノム解析による次世代影響メカニズムの解明である。我々はこの10年間、妊婦のPCBs・ダイオキシン類、有機フッ素化合物（PFCs）、有機塩素系農薬、フタル酸エステル類、ビスフェノールA（BPA）、メチル水銀等の環境化学物質曝露濃度を測定し、その影響について児の追跡を行ってきた。表1に我々がこれまでに測定を行った環境化学物質と甲状腺ホルモン・性ホルモン等の生化学的測定結果についての一覧を示した。表2は我々がこれまでに調べた遺伝子多型の一覧、およびその児への影響について示した。

本稿では、これまでの北海道スタディでの研究成果について紹介する。

札幌（小規模）コーホート（図2）は2002年7月から2005年10月までに札幌東豊病院（札幌市）でリクルートを行い、参加登録した妊娠23-35週の妊婦514名からなる。このコーホートでは母体血中PCBs・ダイオキシン類、PFCs、有機塩素系農薬、BPA、フタル酸エステル類などの多数の環境化学物質曝露濃度について測定を行っており、また生後6・18ヶ月、3才半・7才時には神経発達検査も行っている。一方、北海道（大規模）コーホート（図3）は、2003年2月から2012年5月までリクルートを行い、参加登録した妊娠初期（妊娠13週以前）の妊婦は20,929名になる。このコーホートでは4ヶ月、1・2・4・7・8才時に追加質問表を配布しており、さらに7才時には児の尿およびハウスダストの採取、調査員の自宅訪問が行われている。8才時には面談での発達検査が行われている。

胎児期のPCBs・ダイオキシン類曝露に関しては、我々はこれまでに児の出生時体重・6ヶ月時の神経発達・アレルギーや感染症への影響について検討してきた。表3に母体血中PCBs・ダイオキシン類濃度が児の出生時体重に与える影響、およびその性差について重回帰分析で解析した結果を示した。ダイオキシン類総量はTEQ（毒性等量）で換算した値を使用した。男児において、PCDD総量（TEQ）、PCDD・PCDF総量（TEQ）、ダイオキシン類総量（TEQ）の増加とともに出生時体重は有意に減少した。異性体別で

は、2,3,4,7,8-PeCDF により出生時体重の有意な減少が認められた （-24.5 g, 95 % CI -387.4 to -61.5)。表 4 に胎児期の PCBs・ダイオキシン類曝露が児の 6 ヶ月時の神経発達検査（BSID-II: The Bayley Scales of Infant Development-Second edition）スコアへ与える影響、およびその性差について、重回帰分析で解析した結果を示した。 PCBs・ダイオキシン類曝露が児の psychomotor developmental index （PDI）に与える影響は男児でより顕著であり、1,2,3,7,8,9-HxCDD・1,2,3,4,6,7,8-HpCDD・2,3,7,8-TCDF・1,2,3,7,8-PeCDF・1,2,3,6,7,8-HxCDF などダイオキシン類異性体の濃度の増加とともに PDI スコアは有意に低下した。この結果により、胎児期における低濃度の PCDDs・PCDFs 曝露が、特に男児において、生後 6 ヶ月の神経発達へ負の影響を与えることが示唆された。表 5 には、胎児期の PCBs・ダイオキシン類曝露が臍帯血 IgE レベルに与える影響について、重回帰分析で解析した結果について示した。 PCBs・ダイオキシン類曝露による IgE レベルの低下は男児でのみ有意に認められた （β = -1.01, 95% CI: -1.79 to -0.23)。表 6 は胎児期 PCBs・ダイオキシン類曝露と 18 ヶ月までの中耳炎発症について、多重ロジスティック回帰分析による結果を示した。発症への影響は男児でより顕著で、PCBs・ダイオキシン類曝露が高くなるほど中耳炎発症のオッズ比は増加傾向を示した。また、ダイオキシン類総量（TEQ）を四分位に分けて解析したところ、男児において第一四分位に比べ、第四四分位ではオッズ比は 4.4 倍（95% CI: 1.2 to 16）となった。男児に比べ、女児では PCBs・ダイオキシン類曝露の中耳炎発症への強い影響は見られなかった。

図 4・5 に母の妊娠中喫煙と母の解毒代謝酵素の遺伝子多型が出生時体重・出生サイズへ及ぼす影響について示した。*AHR* 遺伝子の GG 型および *CYP1A1* 遺伝子の TC/CC 型を持つ喫煙者から産まれた児では 315 g の出生時体重の減少、*CYP1A1* 遺伝子の TC/TT 型と *GSTM1* 遺伝子の *null* 型を持つ喫煙者から産まれた児では 237 g の出生時体重の減少が認められた（図 4)。また、*NQO1* 遺伝子の CC 型の喫煙者に産まれた児では、199 g の出生時体重の減少が認められた。

胎児期の有機フッ素化合物（PFCs）曝露に関しては、現在までに児の出生時体重、アレルギー・感染症について影響を検討してきた。また、2003 年から 2009 年まで、各年度 300 名をランダム抽出し、母体血中の 11 種類の PFCs 濃度について測定し、PFCs 濃度の経年変化について検討した。PFOS・PFOA の曝露濃度減少が観察されたが、対して PFNA・PFDA の濃度は年ごとにそれぞれ 4.7%および 2.4%増加の増加が認められた。出生時体重への影響については、母体血中 PFOS 曝露 10 倍ごとに 148.8 g （95% CI: -297 to -0.5）の有意な減少が認められた。またその影響は女児でより顕著で、PFOS 曝露濃度 10 倍ごとに 269.4 g （95% CI: −465.7 to −73.0）の有意な減少が認められた。児の IgE レベルへの影響については、女児において PFOA 曝露の増加とともに臍帯血 IgE レベルは有意に減少した。しかしながら、PFOS・PFOA ともに、胎児期曝露と 18 ヶ月までの食物アレルギー・湿疹・喘鳴・中耳炎発症への有意な影響は見られなかった。

我々のこの 10 年の研究により、 PCBs・ダイオキシン類、PFCs、喫煙等の胎児期曝露が、出生時体重・神経発達・アレルギーなど児の健康に及ぼす数々の負の影響が明らかになった。また、その影響の程度は母親の遺伝子多型により異なり、曝露に対し遺伝的に脆弱な集団の存在が明らかになった。さらに、PCBs・ダイオキシン類曝露の影響に

は性差が存在し、男児でより影響が顕著であること、ダイオキシン類の異性体により影響の程度が異なることが示された。胎児期 PFCs 曝露については、PFOA 曝露により出生時体重が女児でより顕著に低下した。妊娠中の PFOS 曝露は母の脂肪酸組成にも影響を及ぼしており、児の発育のみならず神経発達などへの影響についても解明が求められる。また、2003-2009 年の母体血中 PFCs の経年変化の観察により、POPs に含まれるようになった PFOS・PFOA 濃度については経年減少が認められるのに対し、規制のない PFNA・PFDA 等の炭素鎖の長い PFCs の濃度の経年的な増加が認められた。今後、これらの PFCs の健康影響についてさらに解明する必要があり、環境化学物質が及ぼす広範な児への健康影響について引き続き解明を進める。

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Introduction

In 1997, Theo Colborn et al. [1] warned of the dangers of environmental chemicals, which act as endocrine disruptors and can eventually led to impairments in reproductive capacity. Since that warning, a myriad of animal and epidemiological studies have been conducted to evaluate the adverse health effects of these endocrine-disrupting chemicals (EDCs) [2–4]. Currently, these chemicals are considered to contribute to numerous adverse health effects, including growth retardation of fetuses and

infants and disturbances in neurodevelopment, thyroid, immune and reproductive systems. Additionally, these chemicals may exert genetic or epigenetic effects when metabolized.

On the other hand, in 1986, Barker and Osmond [5] suggested the relationship between poor nutrition in early life and later risk for ischemic heart disease. This observation, which had linked the importance of the intrauterine and early childhood nutritional environment and later disease risk, as well as dozens of additional epidemiological studies suggested a relationship between low birth weight and future risk of certain diseases such as cardiovascular disease, type 2 diabetes, obesity, schizophrenia and asthma. Today, these concepts have been expanded from birth weight to the entire fetal and infantile development, which led to the establishment of the Developmental Origin of Health and Disease (DOHaD) hypothesis [6, 7].

In light of these two groundbreaking concepts, there is a great concern that the consequences of intrauterine growth restriction or intrauterine insults caused by prenatal exposure to the environmental chemicals might linger throughout one's life.

Among the environmental chemicals, two of the most studied substances in environmental epidemiology are polychlorinated biphenyls (PCBs) and dioxins. Thus far, various cohort studies have been conducted to estimate the effects of these substances on fetal and infantile health. These studies inferred that prenatal exposure to PCB/ dioxin could result in fetal growth restriction, cognitive and motor developmental retardation, disrupted sexual dimorphic behavior or reproductive health, and weakened immune systems [8–14].

Furthermore, as a result of recent growing concerns about the adverse health effects of perfluorinated compounds (PFCs), several epidemiological studies were conducted to evaluate the health effects of intrauterine PFCs exposure. Three studies reported correlations between prenatal PFOS/PFOA exposure and reduced birth weight [15–17]. Moreover, in a Danish study, the authors suggested that prenatal PFOA exposure could also increase the risk for obesity and the levels of insulin and leptin in females at 20 years of age [18], which was in line with the Developmental Origin of Health and Disease hypothesis. In addition, recent studies indicated that prenatal exposure to PFCs could also affect fetal and infantile thyroid function [19] as well as the immune system [20, 21].

These adverse health effects were considered to result from the endocrine disrupting activities of the environmental chemicals [3]. However, at this moment, the adverse health effects of prenatal exposures to the environmental chemicals are controversial; despite evidence from animal models, there is insufficient epidemiological evidence to substantiate this assertion [9, 11]. In addition, this assertion is complicated by the fact that the effects of these chemicals in humans are still not fully understood. Many toxicological studies in animals suggest the dose-additive effects of chemicals acting on common endocrine pathways. However, it is challenging to estimate the effects of these compounds in humans that are constantly exposed to a wide variety of chemicals in their daily life [22].

Conversely, it is plausible that the adverse effects of the EDCs are attributed not only to their exposure dosage but also to the activities of the enzymes that metabolize these chemicals in the body and the binding affinity of these chemicals to their receptor, which may result in

inhibition or facilitation of the expression of genes essential for human development. The function and expression levels of proteins are influenced by genetic factors such as single nucleotide polymorphisms (SNPs) and copy number variations (CNVs). Therefore, it is important to uncover genetic risk factors to environmental chemical exposure because there are currently few studies that take these factors into account. Thus, to clarify the effect of prenatal exposure to environmental chemicals on children's health, it is important to study the effects of exposure to EDCs via both genetic and environmental approaches as well as evaluating gene–environment interactions.

One of the examples of the gene–environment interactions is the etiology of hypospadias. Hypospadias is a common congenital anomaly caused by an incomplete fusion of the urethral folds. In our previous studies, we had clarified the etiology of hypospadias with genetic factors that were related to fetal endocrine activity such as the estrogen receptors (ESR1 and 2) and 17b-hydroxysteroid dehydrogenase type 3 (17bHSD3) and maternal hormonal activity such as the cytochrome P450 1A1 (CYP1A1) in a retrospective case–control study [23–26]. Because development of the urethral and external genital system in the male fetus is androgen-dependent, abnormalities in the synthesis and metabolism of androgens resulting from EDCs exposure can result in abnormal genital development phenotypes.

However, there were some limitations in our previous studies. First, in the retrospective case–control study, there was not sufficient evidence to support a causal relationship between hypospadias and the environmental exposures because it was impossible to obtain relevant information about EDCs exposure levels prior to the study baseline. Second, there was both information and selection bias, i.e., recall bias, etc. Thus, we established the first large-scale birth cohort study in Japan in 2002 because of the need for investigating the effects of environmental exposures prospectively combined with genetic predispositions to evaluate the development and health of individuals from the prenatal period to adolescence (up to 13 years old) [26].

This study was primarily concerned with (1) examining the possible negative effects of perinatal environmental chemical exposures on birth outcomes, including congenital anomalies and growth retardation, (2) following the development of allergies, infectious diseases and neurodevelopmental disorders and performing a longitudinal observation of child development, (3) identifying a highrisk group classified by genetic susceptibility to environmental chemicals and (4) identifying the additive effects of various chemicals encountered in the daily environment.

The purpose of this review is to summarize the results of our recent studies and to address the necessary issues to be solved in the future.

Methods

Study areas and subjects

The Hokkaido study on Environment and Children's Health is an ongoing cohort study that began in 2002. The study consists of two prospective birth cohorts: the Sapporo (Toho hospital) cohort with one obstetric hospital in Sapporo City and the Hokkaido (large-scale) cohort with 37 hospitals and clinics in the Hokkaido prefecture. Hokkaido is the northern most prefecture and the second largest island of Japan; it has an area of 83,457 km2, equivalent

to that of Austria. The population of Hokkaido is about 5.4 million, which is similar to that of Finland (Fig. 1).

The enrollment of the Sapporo cohort (Toho hospital) was conducted from July 2002 to October 2005. The subjects were women that were enrolled at 23–35 weeks of gestation and delivered at the Toho hospital. All of the subjects were residents of Sapporo City or surrounding areas.

From February 2003 through March 2012, the Hokkaido (large-scale) cohort had conducted the enrollment of women during early pregnancy (¥13 weeks of gestational age) that visited one of the associated hospitals or clinics in the study area for prenatal health care in the maternity unit. This cohort consists of 20,940 pregnant women. In total, 37 hospitals and clinics in the Hokkaido prefecture participated in the study (the names of the hospitals are listed at the end of the paper). The study was conducted with the informed consent of all subjects in written form. The Institutional Ethical Board for Human Gene and Genome studies at Hokkaido University Center for Environmental and Health Sciences (CEHS) and Hokkaido University Graduate School of Medicine approved the study protocol.

Study design

The protocol for the study that is currently being conducted (subjects, outcomes and exposure measurement items) was partly described in the previous review [27]. In the current paper, the complete study design (up to 8 years), including the continued observation of subjects, has been described in Fig. 2 (the Sapporo cohort) and Fig. 3 (the Hokkaido large-scale cohort).

When examining subjects in the Sapporo cohort (Fig. 2), observations were focused on the association between child growth, neurodevelopment, allergy and infectious diseases, and low-level exposure to environmental chemicals during pregnancy and infancy. In this cohort, a self-administered questionnaire was completed at the time of enrolment to obtain baseline information including parental demographic characteristics, dietary habits including the amount and species of fish consumed, exposure to chemical compounds in their daily life, smoking history, alcohol consumption, caffeine intake and household income. Information on pregnancy complications, gestational age at birth, infant gender and birth size was obtained from maternal and infant medical records.

Follow-up questionnaires were also used at 18 months, 3.5 and 7 years of age to obtain relevant information including allergies, dietary habits and the smoking history of mother and her partner. Additionally, in the follow-up questionnaires, we also obtained information pertaining to the medical history of the children such as atopic dermatitis, asthma, allergies, otitis media, pneumonia or bronchitis and chickenpox. At 18 months and 3.5 years of age, infants were defined as having allergies or an infection if there was a diagnosis from a doctor, the infant was hospitalized or the infant received medical treatment. At 7 years of age, the International Study of Asthma and Allergies in Childhood (ISAAC) criteria was used to determine if the children had allergies or an infection [28]. In addition, we followed the neurodevelopment of the children using several behavioral examinations at 6–7, 18 months, 3.5 and 7 years of age to assess the effect of low-level intrauterine exposure to toxic chemicals on childhood neurodevelopment. More detailed protocols and information regarding

neurodevelopmental examinations are described in the "Outcome Measurement" section below.

The Hokkaido cohort (Fig. 3) was established to assess the prevalence of congenital anomalies including cleft lip and palate, congenital heart defects, hypospadias and cryptorchidism. In addition, this cohort was used to explore the possible causes of these malformations, as well as the prevalence of childhood allergies and neurodevelopmental disorders including Attention Deficit Hyperactivity Disorder (ADHD). In this cohort, a baseline questionnaire survey was conducted at the time of enrollment during the first trimester to obtain parental information such as demographic characteristics, medical and obstetric history, dietary supplement intake during pregnancy, smoking history, alcohol and coffee consumption and chemical exposures at work. Perinatal data such as birth weight, infant gender, mode of delivery, multiple conception and the diagnosis of congenital anomalies were obtained from birth records completed by an obstetrician. We classified 55 congenital anomalies as "representative congenital anomalies" according to the classification by Konishi [29] with some additional anomalies by our study group such as congenital heart diseases, hypospadias, cryptorchidism etc., to study the effect of mutagens and teratogens among EDCs. The first follow-up questionnaire was used on infants at 4 months of age to obtain relevant data including birth size, gestational age at birth and parental smoking history during the second and third trimester. The successive follow-up questionnaires were administered at 1, 2, 4, 7 and 8 years of age to obtain relevant information such as child height and weight measurements obtained at regular health checkups, vaccination history, dietary habits and parental smoking history. In these follow-up questionnaires, we also asked about the medical history of the children. Specifically, we determined if the children developed atopic dermatitis, asthma, allergies, otitis media, pneumonia or bronchitis, chickenpox, heart disease, hypospadias or cryptorchidism, thyroid gland malfunction, epilepsy or developmental disorders. At 1, 2, 4 and 7 years old, allergy and infection information was obtained by using the International Study of Asthma and Allergies in Childhood (ISAAC) [28] and the American Thoracic Society-Division of Lung Disease (ATS-DLD) [30]. At 1 and 2 years of age, infants were defined as having allergies or an infection if there was a diagnosis by a doctor, the infant was hospitalized or the infant received medical treatment. At 8 years old, several examinations for neurodevelopmental disorders were conducted to investigate the effects of perinatal and postnatal chemical exposure on children's health, specifically neurodevelopment.

Specimen collection and biochemical measurements

In the Sapporo cohort, maternal blood samples were collected during late pregnancy, usually after the 30th week of gestation. Cord blood and placenta were taken immediately after birth. Maternal hair samples were also collected within 5 days following delivery, and breast milk from nursing mothers was collected within 4 weeks following birth. In the Hokkaido cohort, maternal blood was collected 3 times: between 6 and 14 weeks of gestational age to represent the organogenetic period, during the third trimester and at delivery. Cord blood was taken immediately after birth in the same manner as done in the Sapporo cohort study.

The items that were measured biochemically

from the specimens are described in Table 1. In the Sapporo cohort, the levels of cord serum immunoglobulin E (IgE) and immunoglobulin A (IgA) were also determined [31]. Thyroid stimulating hormone (TSH) and free thyroxine (FT4) levels of the mother and newborn were measured as part of a mass-screening program conducted in Sapporo City. The levels of 9 key sex hormones in the cord blood (e.g., Estradiol, Testosterone, Progesterone, etc.) and the levels of 11 fatty acids in the maternal plasma were also measured by liquid chromatography-tandem mass spectrometry (LC–MS/MS) and gas chromatography-mass spectrometry (GC–MS), respectively. In the Hokkaido cohort, maternal serum was used to measure folic acid levels [32].

Exposure measurements

PCBs, OH-PCBs and Dioxins

In the Sapporo cohort, the levels of 29 congeners of dioxins and dioxin-like polychlorinated biphenyls (DL-PCBs) [7 polychlorinated dibenzodioxins (PCDDs), 10 polychlorinated dibenzofurans (PCDFs), 4 Non-ortho PCBs and 8 Mono-ortho PCBs], 58 congeners of the other PCBs and 5 congeners of hydroxylated polychlorinated biphenyls (OH-PCBs) in maternal blood and breast milk were measured using a high-resolution gas chromatography/high-resolution mass spectrometer (HRGC/HRMS) at the Fukuoka Institute of Health and Environmental Sciences [33–37]. The Toxicity Equivalency Quantity (TEQ) levels were calculated by multiplying the levels of individual congeners by its toxic equivalency factor (TEF) values of WHO 2005 [38].

PFCs

In the Sapporo cohort, PFOS and PFOA levels in maternal blood, cord blood and breast milk were analyzed by LC– MS/MS at Hoshi University [39, 40]. For the Hokkaido cohort study, among PFCs, 11 perfluoroalkyl acids (PFAAs) [perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), perfluorododecanoic acid (PFDoDA), perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA), perfluorohexane acid (PFHxS) and perfluorooctane sulfonate (PFOS)] were measured in maternal plasma using simultaneous analysis with ultraperformance liquid chromatography in combination with triple quadrupole mass spectrometry (UPLC-MS/MS) at the Research Faculty of Agriculture, Hokkaido University [41].

Organochlorine pesticides

In the Sapporo cohort, the levels of persistent organochlorine pesticides in maternal blood were analyzed by a gas chromatography/negative-ion chemical-ionization mass spectrometry (GC/NCIMS) and a gas chromatography/high-resolution mass spectrometry (GC/HRMS) at IDEA Consultants, Inc. [42].

Metals

In the Sapporo cohort, total mercury levels in maternal hair samples were measured by an oxygen combustion-gold amalgamation method using an atomic absorption detector at the National Institute for Minamata Disease [43, 44].

Phthalate esters and organophosphate flame retardants

In the Sapporo cohort, to determine maternal phthalate exposure levels, MEHP (a metabolite of DEHP) levels in maternal blood were analyzed by GC–MS at Nagoya University [45]. In the Hokkaido cohort, 7 phthalates and 11 organophosphate flame-retardants were measured from dust samples using GC–MS (SIM) analysis. House dust mites were also measured using the ELISA method. The method to analyze 7 phthalate metabolites in urine samples by GC–MS was established, and urine samples from the children were measured to examine the correlation between these metabolites and asthma and allergies [46, 47]. Home visits were also conducted for the children that lived in Sapporo City. During the home visit, house dust and urine samples from the child were collected. In addition, trained researchers evaluated the home interior and dampness.

Bisphenol A

In the Sapporo cohort, Bisphenol A concentrations in maternal and cord blood were analyzed by isotope dilutionliquid chromatography-tandem mass spectrometry (IDLC–MS/MS) at IDEA Consultants, Inc. [48].

Cotinine

In the Sapporo and the Hokkaido cohorts, cotinine concentrations in maternal serum were measured using an enzyme-linked immunosorbent assay (ELISA) kit to evaluate smoking exposure levels [49].

Outcomemeasurements

The Sapporo cohort

In the Sapporo cohort, with the purpose of assessing the neurodevelopment of the children, several behavioral examinations were conducted during each study period. The Bayley Scales of Infant Development second edition (BSID-II) was used at 6–7 and 18 months of age. The Fagan Test of Infant Intelligence (FTII) was performed to measure visual recognition memory and cognitive ability in infants aged 6–7 months. To examine developmental progress, the Japanese version of the Denver Developmental Screening Tests (DDST) was used at 18 months of age. At 3.5 years of age, child and maternal intelligence was measured using the Japanese version of the Kaufman Assessment Battery for Children (K-ABC) and the Wechsler Adult Intelligence Scale-Revised (WAIS-R), respectively. At 7 years of age, the Wechsler Intelligence Scale for Children third edition (WISC-III) and the Wisconsin Card Sorting Test (WCST-KFS version) were used to assess the intellectual development and executive function of the children [50, 51]. The Evaluation of Environmental Stimulation (EES) was used to investigate the environmental conditions of children at 6, 18 months and 3.5 years of age. The Japanese version of the Child Behavior Checklist (CBCL) was used to collect information on child behavior at age 3.5 and 7 years of age. The check list of play behavior, Pre-School Activity InventoryJapanese version (PSAI-J), which was translated from the original version of PSAI, was used to assess the play behavior of the children at 7 years of age [52]. In addition, we also obtained the children's medical history from the follow-up questionnaires performed at each study period. The children's medical history contained information pertaining to the development of atopic dermatitis, asthma, allergies, otitis media, pneumonia or bronchitis and chickenpox.

The Hokkaido cohort

In the Hokkaido cohort, the development of allergies at 1, 2, 4 and 7 years of age and neurodevelopmental disorders at 8 years of age were examined in detail. For allergy assessment, follow-up questionnaires were distributed to children aged 1, 2, 4 and 7 years old, which included questions pertaining to asthma and allergies from the ISAAC and ATS-DLD questionnaires [28, 30]. We also obtained the medical history of the children from the follow-up questionnaires during each study period. The medical histories contained information pertaining to the development of atopic dermatitis, asthma, allergies, otitis media, pneumonia or bronchitis, chickenpox, heart disease, hypospadias or cryptorchidism, thyroid gland malfunction, epilepsy and developmental disorders. In addition to the questionnaire survey, mothers were asked to collect house dust and a sample of the child's urine when the child reached 7 years old.

At 8 years of age, a specific follow-up questionnaire was used to assess the development of neurodevelopmental disorders, specifically ADHD. The questionnaire contained questions pertaining to health status including the treatment the subject received for ADHD, the hours of rising and bedtime as a daily rhythm, and the number of hours the subject enjoys audio-visual tools. To assess ADHD, the Conners third Edition-Parent Japanese version (Conners3P) and the ADHD Rating Scale-IV (ADHD-RS-IV) were used. We also used the Pre-School Activities Inventory Japanese version (PSAI-J) to assess the play behavior of the children. The Evaluation of Environmental Stimulation (EES) was used as a questionnaire to assess the subject's home environment [53]. We also assessed any stressful life events of the children by using the Life Event Questionnaire for Parents (Life Event) [54].

After receiving responses from the 8-year questionnaire, additional questionnaires were distributed to collect more information about the family. The additional questionnaire assessed the working status and health of the parents, the mental condition of the mother, and the use or lack of use of supportive education. To assess a child-rearing environment, we asked about the parent's social networks and supports during child rearing. To assess developmental disorders such as Autism and Asperger syndrome, we used the Japanese version of the Autism screening Questionnaire (ASQ). Additional assessments of the children we obtained using the Japanese version of the Child Behavior Checklist (CBCL) and The Wechsler Intelligence Scale for Children fourth edition (WISC-IV). We also used the Parenting Stress Index (PSI) in Japanese.

Genetic analyses

Genes that were already analyzed using the SNP assay are described in Table 2. Genetic polymorphisms were determined by means of the Taq Man (Applied Biosystems, Inc., Foster City, CA, USA) polymerase chain reaction (PCR) method using minor groove binder (MGB) probes. The polymorphisms analyzed thus far are rs4646903 (T [C, MspI] and rs1048963 (A [G, Ile462Val] of CYP1A1 (cytochrome P450, family 1, subfamily A polypeptide 1), rs762551 (A [C] of CYP1A2 (CYP1 subfamily A polypeptide 2), rs1056836 (C [G, Leu432Val] of CYP1B1 (CYP1 subfamily B polypeptide 1), rs2066853 (G [A, Arg554Lys] of AHR (aryl hydrocarbon receptor), rs2292596 (C [G, Pro185Ala] of AHRR (AHR repressor), rs1800566 (C609T) of NQO1 (NAD(P)H: quinone oxidoreductase 1), rs3813864 (-1294G/C) of CYP2E1 (CYP2

subfamily E polypeptide 1), rs1801133 (C677T) and rs1801131 (A1298C) of MTHFR (methylenetetrahydrofolate reductase). In addition, copy number variations (CNVs) in GSTM1 (glutathione S-transferase mu-1) and GSTT1 (glutathione S-transferase theta-1) were also evaluated [55–58].

Results

The characteristics of the participants of the Hokkaido study

A total of 514 mothers were registered in the Sapporo cohort, and another 20,940 mothers were registered in the Hokkaido cohort as of the end of April 2012. The profile of the Sapporo cohort and the partial profile (n = 2,777) of the Hokkaido cohort had been described previously [27]. We also estimated the prevalence of congenital anomalies in the Hokkaido prefecture. Among the 19,680 mothers included in the Hokkaido cohort between 2003 and 2012, there were 378 subjects with congenital anomalies. The most frequent congenital anomaly was congenital heart defects (35.6 per 10,000 persons), followed by cryptorchidism (15.2), down syndrome (12.2), polydactyly (9.7), hypospadias (9.1) and hydronephrosis (7.6). The total prevalence of congenital anomalies was similar to nationwide data reported by the Japan Association of Obstetricians and Gynecologists (JAOG). However, the number of serious cases was less than that of the JAOG since the members of the JAOG are medical universities and tertiary hospitals and they tend to treat pregnant women with severe complications including fetal congenital anomalies, whereas those of our cohort study are general hospitals and clinics.

The effects of PCDD/PCDF and dioxin-like PCB exposure

Birth weight

In the Sapporo cohort, we observed significant negative correlations between the birth weight of all infants and total PCDF levels, total PCDD TEQ, total PCDF TEQ, total PCDD/PCDF TEQ and total TEQ levels in maternal blood during pregnancy after adjustment for potential covariates. Among male infants, significant adverse associations between birth weight and total PCDD TEQ levels, total PCDD/PCDF TEQ levels and total TEQ levels were found. Moreover, we found significant negative association between birth weight and the levels of 2,3,4,7,8-PeCDF (-24.5 g, 95 % CI -387.4 to -61.5) [59] (Table 3).

Neurodevelopment

In the Sapporo cohort, after adjusting for potential confounding variables, total PCDD, total PCDDs/PCDF and 1,2,3,4,6,7,8-HpCDD levels in maternal blood during pregnancy were significantly negatively associated with the mental developmental index (MDI) of BSID-II at 6 months of age. Total 1,2,3,7,8,9-HxCDD, 1,2,3,4,6,7,8-HpCDD, 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF and 1,2,3,6,7,8-HxCDF were significantly negatively associated with the psychomotor developmental index (PDI) of BSID-II at 6 months of age. Our results suggest that a low-level of exposure to several congeners of PCDDs or PCDFs during pregnancy can affect the neurodevelopment of 6-month-old infants [60]. In addition, when we stratified the data by infant sex, the effects of intrauterine exposure to select PCDD, PCDF and PCB congeners on the PDI score in male infants were more significant (Table 4).

Allergy and infectious diseases

In the Sapporo cohort, our results show that dioxins concentrations in maternal blood during pregnancy are only negatively correlated with cord serum IgE levels in male infants [61] (Table 5). Relatively higher levels of PCDFs were associated with a significantly increased risk of otitis media at 18 months of age, among all infants (odds ratio = 2.5, 95 % confidence interval = 1.1–5.9). Relatively higher levels of 2,3,4,7,8-PeCDF were also associated with a significantly increased risk of otitis media (odds ratio = 5.3, 95 % confidence interval = 1.5–19) among male infants (Table 6). However, we observed a weak association between dioxin-like compound levels and allergy symptoms during infancy. At environmental levels, prenatal exposure to dioxin-like compounds may alter immune function and increase the risk of infections in infancy, especially among males. The compound 2,3,4,7,8-PeCDF may be responsible for this [62].

The effects of PFCs exposure

Temporal trends of PFC levels in maternal plasma

In the Sapporo cohort, the concentrations of PFOS and PFOA ranged from 1.3 to 16.2 ng/ml for PFOS and from below the detection limit to 5.3 ng/ml for PFOA (both detection limits were 0.5 ng/ml) in the blood of pregnant women recruited between 2002 and 2005 [63].

In the Hokkaido cohort, between February 2003 and December 2009, 300 women were randomly selected every year, and the concentrations of 11 PFCs were measured in 2,095 maternal plasma samples. A temporal trend in PFC levels from 2003 to 2011 was also examined. The PFOS and PFOA concentrations in the Hokkaido cohort were lower than those of pregnant women in the Sapporo cohort. Additionally, PFUnDA, PFDoDA and PFTrDA levels were higher in the Hokkaido cohort than individuals of foreign countries. Although the values were lower than the values obtained from individuals in other areas of Japan, there was no significant temporal trend [64].

Birth weight

We examined a correlation between maternal serum PFOS and PFOA concentrations and infant birth weight in the Sapporo cohort. A log10-unit increase in PFOS levels correlated with a decrease in birth weight of 148.8 g (95 % CI 297.0–0.5) after adjusting for confounders; however, no correlation was observed between PFOA levels and birth weight. Our results indicate that in utero exposure to relatively low levels of PFOS is negatively correlated with birth weight [63].

In the Hokkaido cohort, the effects of 11 PFCs including PFHxA, PFHpA, PFHxS, PFOS, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA and PFTeDA in maternal blood obtained during pregnancy were evaluated. After adjusting for possible confounding factors, PFNA levels negatively correlated with birth weight (per ln-unit: partial regression coefficient b = -41.7 g, 95 % CI, -77.9 to -5.6 g). After gender stratification, PFNA levels negatively correlated with male birth weight (per ln-unit: b = -59.3 g, 95 % CI, -110.2 to -8.3 g). Additionally, PFUnDA and PFTrDA levels negatively correlated with female birth weight (per ln-unit: b = -42.0 g, 95 % CI, -84.6 to 0.6 g and b = -44.9 g, 95 % CI, -90.1 to 0.3 g, respectively).

Allergy and infectious diseases

In the Sapporo cohort, we investigated the relationship between prenatal exposure to PFOS and PFOA and the development of infant allergies and infectious diseases during the first 18 months of life. Additionally, the effects of PFOS and PFOA on cord blood IgE levels were also evaluated. We found a curvilinear relationship between maternal PFOA levels and cord blood IgE levels. Cord blood IgE levels decreased significantly with high maternal PFOA concentrations among female infants. When log10-transformed maternal PFOA levels changed from 0.3 to 0.7 ng/mL, log10-transformed cord blood IgE levels greatly decreased by -0.863 IU/mL. However, there were no significant associations among maternal PFOS and PFOA levels and food allergies, eczema, wheezing or otitis media in the 18-month-old infants after adjustment for potential confounding variables [31].

In the Hokkaido cohort, we investigated the relationship between prenatal exposure to 11 PFCs and infant allergies during the first 12 months of life. The characteristics of the participants and information pertaining to infant allergies were obtained from a baseline questionnaire administered to the mother during pregnancy, medical records from the time of delivery and a followup questionnaire when the child was 12 months of age. The risk of eczema, wheezing and food allergies during the first 12 months of life was not associated with maternal levels of 11 PFCs, including longer-chain compounds. Odds ratios for eczema and wheezing ranged from 0.66 to 0.73 and from 0.60 to 0.81 for the three higher quartiles of maternal PFTrDA levels, compared with the lowest in the adjusted models, but no dose– response pattern was found [65].

Gene–environment interaction

The effects of maternal genetic polymorphisms on dioxin concentration

Dioxins are metabolized by cytochrome P450, family 1 (CYP1) via AHR. We determined whether different blood dioxin concentrations are associated with polymorphisms in the AHR (dbSNP ID: rs2066853), the AHRR (rs2292596), the CYP1A1 (rs4646903 and rs1048963), the CYP1A2 (rs762551) and the CYP1B1 (rs1056836) in pregnant Japanese women. Comparisons between the GG, GA and AA genotypes of the AHR showed a significant difference for both the mono-ortho PCBs concentrations (genotype model; GG:GA:AA = 11,266.3:13,146.5:12,948.9 (pg/g lipid), p = 0.016) and that of toxicity equivalence quantities [TEQs] (GG:GA:AA = 0.338:0.394:0.388 (TEQ pg/g lipid), p = 0.016). Second, we found a significant association with the dominant genotype model for the PCDDs TEQs ([TT ? TC]:CC = 7.408:6.480 (TEQ pg/g lipid), p = 0.048) and for PCDFs TEQs ([TT ? TC]:CC = 2.596:2.267 (TEQ pg/g lipid), p = 0.035) of CYP1A1 (rs4646903). No significant differences were found among blood dioxin concentrations and polymorphisms in AHRR, CYP1A1 (rs1048963), CYP1A2 and CYP1B1. Thus, polymorphisms in AHR and CYP1A1 (rs4646903) were associated with maternal dioxin concentrations [55].

Genetic polymorphisms and maternal smoking

The effects of maternal smoking and genetic polymorphisms on infant birth size were examined in the Sapporo cohort. Birth weight and length were significantly lower among infants born to smokers with the AHR GG genotype, the CYP1A1 TC/CC genotype or the GSTM1 null genotype. When combinations of

these genotypes were considered, birth weight and length were significantly lower for infants of continuously smoking women with the AHR GG genotype and CYP1A1 TC/CC genotype (-315 g and -1.7 cm, respectively) and with the CYP1A1 TC/CC genotype and GSTM1 null genotype (-237 g and -1.3 cm, respectively) [56] (Fig. 4). For polymorphisms in the gene-encoding N-nitrosamine-metabolizing enzymes, NQO1, birth weight, birth length and birth head circumference were significantly reduced (-199 g, -0.8 cm and -0.7 cm, respectively) among infants born to smokers with the NQO1 CC genotype (Fig. 5). This genotype did not confer adverse effects among women who had never smoked or who quit smoking during the first trimester. Our results suggest an important modifying role of polymorphisms in metabolizing enzyme genes in concert with the adverse effects of maternal smoking on infant birth size [57].

Folate, maternal smoking and genetic polymorphisms

Folate is essential for fetal growth and development, and smoking has been associated with nutritional deficiencies in vitamins including folate. The birth weight of infants born to moderate smokers (≥10 cigarettes per day) with low folate status (<6.0 ng/ml) was lower by 107 g compared with non-smokers having a normal folate status (≥6.0 ng/ml). Maternal 5,10-methylenetetrahydrofolate reductase (MTHFR) 1298AA was associated with low folate status. The 5,10-MTHFR AA genotype was associated with a decrease in birth weight by 107 g in infants born to smokers. After stratification by infant gender, the effect was more pronounced in male infants with a reduction in birth weight of 117 g. Female infants never demonstrated any statistically significant changes in birth weight [58].

Discussion

What are the primary strengths and weaknesses of the study?

The design of our study is a prospective cohort study intended to collect data on environmental exposures during fetal development and to control for potential confounders. The detailed measurements in exposures and outcomes are adequate to detect the various effects of perinatal environmental and genetic determinants on childhood outcomes. In the Sapporo cohort study, face-to-face examinations for neurodevelopment assessment were conducted. The Hokkaido cohort had been the largest birth cohort in Japan until 2011 when the nation-wide cohort study, the Japan Environment and Children's Study (JECS), was launched based upon our study design. A potential problem of our study is that both the Sapporo and Hokkaido cohorts may have been biased in participant selection because they are both hospital-based studies, although the latter cohort consists of the hospitals and clinics over the Hokkaido areas to mitigate that bias (Fig. 1). In addition, despite our efforts to keep track of participants' residence with periodical newsletters, some levels of attrition were caused by individuals moving outside of the study area.

The main findings of the study

Over the last decade, we have been intensely investigating the effects of intrauterine chemical exposures on children's health. The main findings of our study are as follows.

1. The effects of dioxins—with emphasis on gender differences