Effects of prenatal perfluoroalkyl acid exposure on cord blood *IGF2/H19* methylation and ponderal index: the Hokkaido study

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Running title. Prenatal PFAAs exposure and *IGF2/H19* methylation

Abstract

Prenatal exposure to perfluoroalkyl acids (PFAAs) influences fetal growth and long-term health. However, whether PFAAs affect offspring DNA methylation patterns to influence health outcomes is yet to be evaluated. Here, we assessed effect of prenatal PFAA exposure on cord blood *insulin-like growth factor 2 (IGF2)*, *H19,* and *LINE1* methylation and its associations with birth size. Mother-child pairs (N = 177) from the Hokkaido Study on Environment and Children's Health were included in the study. Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) levels in maternal serum were measured by liquid chromatography-tandem mass spectrometry. *IGF2*, *H19,* and *LINE1* methylation in cord blood DNA was determined by pyrosequencing. After full adjustment in multiple linear regression models, *IGF2* methylation showed a significant negative association with ln–unit increase in PFOA (partial regression coefficient = -0.73, 95% confidence interval: -1.44 to -0.02). Mediation analysis suggested that reduced *IGF2* methylation explained approximately 21% of the observed association between PFOA exposure and reduced ponderal index of the infant at birth. These results indicated that effects of prenatal PFOA exposure could be mediated through DNA methylation. Further study will be required to determine the potential for long-term adverse health effects of reduced *IGF2* methylation induced by PFOA exposure.

Key words: epidemiology; perfluorinated chemicals; child exposure/health; endocrine disruptors; DNA methylation; birth cohort

Introduction

Perfluoroalkyl acids (PFAAs) are synthetic compounds widely used in the manufacture of consumer goods as well as in numerous industrial applications. Since the classification of perfluorooctane sulfonate (PFOS) within Annex B of the Stockholm Convention on Persistent Organic Pollutants in 2009, the use of PFOS as well as perfluorooctanoic acid (PFOA) has been diminishing worldwide (1, 2). However, PFAAs remain widely distributed and abundant throughout the environment owing to their original prevalence and slow degradation rates (3). They do not readily metabolize and exhibit long half-lives: 5.4 years for PFOS and 3.8 years for PFOA in humans, which can result in bioaccumulation (4). Owing to the placental transfer capability of PFAAs (5, 6), fetuses can be exposed to these compounds *in utero*. Studying the effects of prenatal exposure to PFAAs on fetal development is therefore warranted.

Earlier epidemiological studies have reported reductions in birth size associated with prenatal PFAAs exposure (7, 8). We have reported, for example, a 269.4 g reduction in birth weight associated with a $\log_{10}$-unit increase of maternal PFOS concentrations among female infants (9). In the same cohort from that study, we observed that maternal PFOS concentrations during pregnancy are negatively associated with triglyceride and essential fatty acids levels in the pregnant women (10). We also demonstrated that prenatal exposure to PFAAs could result in disruption of thyroid (11) and reproductive hormone balance (12) in

infants and mothers. Thus, one explanation for the effects of PFAAs on birth size is induction of maternal and fetal physiological changes, including altered metabolic or hormone endpoints.

Another possible explanation is that prenatal PFAAs exposure modifies the epigenetic machinery of developing fetuses leading to changes in gene expression and subsequent phenotypes. DNA methylation is an epigenetic process that plays a role in embryonic development and cellular differentiation. It occurs by addition of a methyl group to a cytosine at cytosine–guanine dinucleotide (CpG) loci and acts like a switch on gene expression (13). This DNA methylation is heritable through cell division but can also be affected by the external environment and has therefore been postulated as a mediator of intrauterine environmental influences on postnatal phenotypes (14).

Previous epidemiological studies have suggested an association between some prenatal environments and cord blood DNA methylation at repetitive sequences such as the long interspersed element 1 *(LINE1)* (15). *LINE1* is globally distributed comprising approximately 17% of the human genome. Methylation of *LINE1* is correlated with DNA methylation across the entire genome and can be used as a surrogate for global DNA methylation (16). The insulin–like growth factor 2 (*IGF2)/H19* locus is also one of the most studied regions in epigenetics. IGF2 is a peptide hormone essential for fetal growth and is expressed from early embryonic stages throughout fetal development. Expression of this

imprinted gene is controlled by DNA methylation at the *IGF2* differentially methylated region (DMR) and *H19* DMR, which are reciprocally methylated and expressed between maternal and paternal alleles (17). It has been suggested that, in addition to its biological function, *IGF2* methylation may serve as an epigenetic marker for intrauterine disruption. Through epidemiological studies, reduced *IGF2* methylation in cord blood or placental tissues has been correlated with reduced fetal growth features such as lower birth weight and small-for-gestational age (SGA) (18, 19). Emerging evidence has indicated a role for the *IGF2* gene in metabolic disorders such as obesity and hypertension in humans (20). One report has suggested that *IGF2/H19* methylation at birth is linked to the development of obesity in early childhood (21).

Whether levels of PFAAs can influence infant health effects through an epigenetic mechanism has not been rigorously evaluated in epidemiological studies. In an earlier study, Guerrero-Preston and colleagues observed that cord blood PFOA concentrations negatively correlated with cord serum global DNA methylation (22). However, this study was cross-sectional with a limited sample size (N=30) and could not control for multiple potential confounders. Furthermore, given that the epigenetic machinery may work in a site- and gene-specific manner, the effects of PFAAs on gene-specific as well as global DNA methylation need to be evaluated. These evaluation of the effects of PFAAs on both global and gene–specific methylation and their association with health outcomes at birth based also

necessitate larger sample sizes than the above earlier investigation.

We hypothesized that prenatal exposure to PFAAs modifies infant DNA methylation in genes essential for fetal growth, which in turn contributes to reduced birth size. In this study, we evaluated the effects of prenatal PFOS and PFOA exposure on gene-specific *IGF2/H19* methylation as well as *LINE1* methylation (as a surrogate for global DNA methylation), and their association with birth weight, length and ponderal index.

Materials and Methods

Study population. Study participants were enrolled as a part of the Hokkaido Study on Environment and Children's Health, an ongoing birth cohort study. Details of the study design were described previously (23, 24). Briefly, pregnant women at 23-35 weeks of gestation were recruited at Toho Hospital, Sapporo, Japan between 2002 and 2005. Of 1796 pregnant women initially approached, the following were excluded from recruitment: registrants of the Japanese cord blood bank (25 %) or women planning to deliver at another hospital (3 %). Subsequently, 514 women agreed to participate in this study (29 %). Of those, based on pre-established criteria, 10 were later excluded due to miscarriage or stillbirth (N = 2), relocation (N = 1), voluntary withdrawal (N = 7). Of 504 subjects whose birth records and baseline questionnaires were available, we further excluded multiple births (N = 7). Subsequently, a total of 441 maternal blood samples and 267 cord blood samples were

available for PFAA and DNA methylation analyses. Among subjects who could provide both maternal and cord blood samples, we excluded mothers whose blood was obtained after delivery (N=58). Overall, 177 mother-infant pairs were evaluated in this study (Fig S1).

The study was conducted after written informed consent was received from all subjects. The institutional ethical board for human gene and genome studies at Hokkaido University Center for Environmental and Health Science and Hokkaido University Graduate School of Medicine approved the study protocol.

Data collection. At enrollment, a self-administered questionnaire was used to obtain baseline information including parental demographic characteristics, anthropometric measurements, and lifestyle factors such as maternal smoking and alcohol consumption (9). Information on pregnancy complications, gestational age, infant gender, and birth weight and birth length was obtained from medical records.

Specimen collection. A 40 mL aliquot of maternal blood samples was collected from participants between 24-41 weeks of gestational age. Cord blood samples were taken immediately after birth. All specimens were stored at -80°C prior to analysis.

Exposure assessment. Detailed methods for the measurement of PFOS and PFOA have been previously described (5, 25). Briefly, serum samples (0.1 mL) were mixed with 0.2 mL internal standard solution containing acetonitrile, centrifuged at 1450 × g for 10 min, and the supernatant was then transferred to a polypropylene tube. An aliquot of the filtered

sample solution was subjected to column-switching liquid chromatography-tandem mass spectrometry (LC–MS/MS). PFOS levels were detected in all samples. PFOA levels for 10 samples (5.6 %) were below the detection limit (0.50 ng/mL). The percentage of samples below the detection limit was small enough to yield fair estimates with a simple substitution approach (26, 27). Therefore, for samples below the detection limit, we assigned a value of half the detection limit (0.25 ng/mL).

Quantification of DNA methylation. Genomic DNA was extracted from umbilical cord blood using a Maxwell® 16 DNA Purification Kit (Promega, Madison, WI, USA). DNA (500 ng) was subjected to a bisulfite conversion using Epitect Plus Bisulfite Kit (Qiagen, Venlo, Netherlands) to convert unmethylated cytosine to uracil, leaving the methylated cytosine unaltered. Bisulfite pyrosequence was performed as previously described (28, 29). We evaluated two *IGF2/H19* regulatory regions; the first was two CpG sites comprising the intragenic *IGF2* differentially methylated region 0 (DMR0), and the other was four CpG sites within *H19* DMR. For *LINE1*, three CpG sites at the promoter region were quantified. Each locus was amplified using HotStarTaq DNA polymerase in PyroMark PCR Kit (Qiagen). Biotin-labeled single-stranded amplicons were bound to the Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden) and purified using the Pyromark Q24 Work Station (Qiagen). Pyrosequencing was performed using Pyromark Q24 system and data was analyzed using Pyro Q-CpG Software (Qiagen). PCR primers, conditions, and sequencing

primers are described in Table S1. All the investigators were blinded to the individual's information related to the study at the time of the experiment. All samples were analyzed in duplicate. Since methylation levels of each site in the given three regions were highly correlated, we averaged the methylation levels of each region (*IGF2*, *H19*, and *LINE1*) and used the average in subsequent analyses.

Data Analysis. The Spearman's correlation test, Mann-Whitney U-test, and Kruskal–Wallis test were applied to determine whether maternal and offspring characteristics were associated with PFAAs exposure. Multiple linear regression analyses were performed to determine associations between maternal PFAA concentrations and infant methylation levels at each locus. Because of skewed distributions, we transformed the PFOS and PFOA concentrations in maternal serum to the natural log (ln) scale. Maternal blood sampling period for PFAA measurement was categorized into three groups, 23-31 weeks, 32-34 weeks, and 35-41 weeks of pregnancy. Multiple linear regression analyses were applied to estimate the effects DNA methylation on infant birth weight, length, and ponderal index, which was calculated as the ratio of birth weight (kg) to cubed birth length (m^3) (30). One subject was excluded from the analysis of birth length and ponderal index *post hoc* because its birth length departed from the mean by more than four standard deviations (SDs), despite its gestational age and birth weight being within normal range. Covariates were selected based on associations between dependent and independent variables observed in our data or

previous studies: in DNA methylation models, we adjusted for maternal age, maternal education, infant sex, maternal smoking during pregnancy, and blood sampling period; in birth size models, we adjusted for gestational age, maternal age, pre-pregnancy BMI, parity, maternal education, maternal smoking during pregnancy, and infant sex. We additionally implemented directed acyclic graphs (DAGs) using *DAGitty* ver. 2.3 (31) for covariate selection, and confirmed that the covariates selected above were neither colliders nor intermediates. As Bartlett's test verified the assumption of equal variance among groups, in quartile models, PFOS and PFOA concentrations were divided into quartiles and a test for a linear trend was performed by linear contrast. Adjusted least square means of methylation levels in each quartile were compared by the Hsu-Dunnet's method accounting for multiple comparisons. For calculation of p for trend, we used linear contrast coefficients -3, -1, +1, +3 assigned to quartiles 1, 2, 3, and 4, respectively (10, 32). Mediation analysis was performed by PROCESS, a macro implemented in SPSS (33). It estimates how an effect of *X* (independent variable) can be apportioned into its indirect effect on *Y* (dependent variable) through *M* (mediator), and its direct effect on *Y*. The indirect effect of *X* on *Y* through *M* can be quantified as the product of two coefficients: *a* (the effect of *X* on *M*) and *b* (the effect of *M* on *Y*) (i.e. *ab*). The bias-corrected and accelerated confidence intervals (BCa CIs) of the indirect effect (*ab*) are calculated by bootstrapping with 1,000 iterations. The effect size of the mediator is calculated using percent mediation (P_M) method (34). Proportion of variance

in *Y* explained by the indirect effect was calculated as $R^2_{med} = r^2_{MY} - (R^2_{Y.MY} - r^2_{XY})$ (35). We further considered whether the three main sources of potential bias in mediation analyses, namely (i) mediator-outcome confounding, (ii) exposure-mediator interaction and (iii) mediator-outcome confounding affected by the exposure (36), were introduced to our observation; we found that only sex could be a confounding factor for mediator-outcome association and therefore we included sex in our mediation model. Statistical analyses were conducted using JMP pro 11 (SAS Institute Inc., NC, USA) and SPSS ver. 22.0 (IBM, NY, USA). P-values of less than 0.05 (two-sided) were considered statistically significant.

Results

Among the 177 mother-infant pairs who were included in the study, mean methylation levels (±SD) for *IGF2* DMR0, *H19* DMR and *LINE1* were 48.5% (±3.0), 52.3% (±2.0) and 75.8% (±1.1), respectively. Geometric means (GM) (25^{th} to 75^{th} percentiles) of PFOS and PFOA concentrations in maternal blood were 5.2 ng/mL (3.9 to 7.2) and 1.3 ng/mL (0.9 to 2.1), respectively (Table 1). Maternal and infant characteristics and their relationship to PFOS and PFOA concentrations are described in Table 2. Maternal and infant characteristics that showed a significant association with both PFOS and PFOA concentrations included parity ($p < 0.01$) and blood sampling period ($p < 0.05$), and with PFOA only, maternal education ($p = 0.03$).

Table 3 shows the results of the multiple linear regression analyses for the effects of PFOS and PFOA on DNA methylation in cord blood. After adjustment for potential confounders, we observed a significant decrease in *IGF2* methylation with a ln-unit increase in PFOA [partial regression coefficient (β) = -0.73, 95% confidence interval (CI): -1.44 to -0.02]. No significant association was observed for PFOS. Similarly, no significant associations were observed between *H19* and *LINE1* methylation with either PFOS or PFOA concentrations.

Figure 1 shows the changes in adjusted least square means of *IGF2* methylation among PFOS and PFOA quartiles. After full adjustment, we observed a decreasing trend of *IGF2* methylation as quartile of PFOA increases (p for trend = 0.007). In addition, with marginal significance, we observed a 1.44% decrease of *IGF2* methylation at the fourth quartile (> 2.1 ng/mL) of PFOA compared with the first quartile (β = -1.44, 95% CI: -2.98 to 0.10, p = 0.074) (Table S3). PFOS quartiles did not show any significant trend on *IGF2* methylation (*p* for trend = 0.29).

We next analyzed whether DNA methylation at *IGF2* was associated with fetal growth (Table 4). After full adjustment, *IGF2* methylation showed a significant association with ponderal index at birth (β = 0.17, 95% CI: 0.06 to 0.27), but not with birth weight or birth length. There was a significant negative association between ponderal index and a ln-unit increase in PFOS concentration after adjustment for potential confounders (β = -1.07,

95% CI: -1.79 to -0.36) (Table 5). We also observed an association between PFOA and ponderal index, however the association was weak and non-significant in a full-adjusted model (β = -0.44, 95% CI: -0.99 to 0.12). Mediation analysis (Figure 2) indicated that there was a significant indirect effect of *IGF2* methylation on the relationship between PFOA exposure and lower ponderal index at birth (*ab* = -0.11, BCa CI [-0.30, -0.02]). Results showed that the mediator (*IGF2* methylation) could account for approximately 21% of the total effect (P_M = 0.21), which explains 1.2% of variance in ponderal index (R^2_{med} = 0.012). *H19* methylation did not show any significant relationship with these fetal growth indices.

Discussion

Our results suggested that prenatal PFOA exposure resulted in a decrease in *IGF2* methylation in cord blood. Although the observed negative association between PFOA exposure and ponderal index at birth did not reach statistical significance at the α = 0.05 threshold, our mediation analysis suggested that reduced *IGF2* methylation can act as a mediator between PFOA exposure and reduced ponderal index, explaining approximately 21% of its total effect. We did not find any significant association between PFAAs exposure and *H19* or *LINE1* methylation.

Previous epidemiological and animal studies have reported a negative association between prenatal PFAA exposure and reduced birth size (7, 8); however, the mechanisms by

which PFAAs affect fetal growth still require elucidation. An original finding of this current study is the involvement of *IGF2* methylation in the effect of prenatal PFOA exposure on ponderal index at birth. To date, only a small number of epidemiological studies have investigated the effects of PFAAs on DNA methylation, and these have focused on global DNA methylation, rather than gene-specific methylation as in this study. Two studies on adult populations have suggested the possibility of an epigenetic effect of PFAAs on global methylation. In the C8 Health Project, Watkins and colleagues studied 685 adult participants who lived in a PFAA-contaminated area. The authors found a significant monotonic increase in *LINE1* methylation across PFOS and PFNA tertiles in the peripheral blood leucocytes (37). Leter and colleagues studied 262 fertile men from three independent populations in Greenland and Eastern Europe. When the three populations were analyzed independently, the authors found a significant association between PFAAs and *LINE1* methylation as well as total cytosine methylation in sperm DNA. However, when the data was analyzed across the total combined population, no significant associations between PFAA exposure and DNA methylation were observed (38).

Prior to this study, there was only one small cross-sectional study that had evaluated the effect of prenatal PFAA exposure on offspring DNA methylation (22). This was conducted by Guerrero-Preston and colleagues who investigated 15 pairs of newborns of smoking mothers and mothers no history of smoking. They demonstrated that cord blood

PFOA concentration was negatively correlated with cord serum global methylated cytosine as determined by enzyme-linked immuno-sorbent assay (ELISA).

As noted earlier, the above studies focused on global DNA methylation, rather than gene-specific methylation and neither did they evaluate whether methylation changes induced by PFAAs exposure could influence health outcomes such as birth size reduction. In this context, it should be noted that the association between birth size and *IGF2/H19* methylation has been relatively well studied. Previous epidemiological studies have reported that reduced *IGF2* methylation in cord blood and placental tissues is correlated with reduced fetal growth such as lower birth weight or small-for-gestational age (SGA) (18, 19). Our study, which to our knowledge, is the first to report that prenatal PFOA exposure results in reduced cord blood *IGF2* methylation, which in turn is associated with decreased ponderal index at birth, is therefore consistent with these observations.

Mediation analysis further confirmed that *IGF2* methylation as a mediator can account for approximately 21% of the total effect of PFOA exposure on ponderal index at birth, which explains 1.2% of variance in ponderal index. It is remarkable that around one-fifth of the effects of prenatal PFOA exposure on reduced ponderal index can be explained by methylation at only one gene, *IGF2*. However, we only evaluated DNA methylation at three regions, and it is possible that methylation of other unmeasured genes are also responsible for the mediatory effect that we observed. Future epigenome-wide study

would reveal other genes that mediate the effects of prenatal PFOA exposure. Simultaneously, ponderal index is an indicator of fatness and measures the relative soft tissue mass to bone structure. Reduced fetal growth may lead to future health problems such as obesity, hypertension, type II diabetes, and associated disorders. Given the long-term effect of DNA methylation at birth on long-term phenotypic alteration, a study following the participants in our birth cohort who were exposed to PFOA *in utero* is warranted.

In our mediation analysis, we found that *IGF2* methylation had a significant mediatory effect on the relationship between PFOA and ponderal index, despite the negative association between PFOA and ponderal index did not meet statistical significance (β = -0.44, 95% CI: -0.99 to 0.12, p = 0.123). In a previous cross-sectional study, Apelberg and colleagues reported a negative association between cord serum PFOA concentration and ponderal index at birth among 293 newborns (39). The cord serum median concentration of PFOA in that study was 1.6 ng/mL, which was similar to or slightly higher than the exposure levels in our study, In addition, the sample size in that study was more than 1.5 times larger than that of our study. Therefore, it is possible that we did not find a significant association between PFOA and ponderal index owing to the limited sample size of our study. Additionally, in cases of mediation analyses, it is possible for an exposure to exert an effect on an outcome indirectly through a mediator, even if we do not observe a significant exposure-outcome association. This can be because of a presence of multiple indirect

pathways or subpopulations that mask true association (33). Further study with a larger sample size will clarify the association between PFOA exposure and lower ponderal index at birth.

We did not find a significant association between prenatal PFAAs exposure and *LINE1* global methylation. This is consistent with two *in vitro* studies on murine and human cells that showed no significant effects of PFOA exposure on global methylation determined

measured by flow cytometric immunodetection and LC-MS/MS (40, 41). As mentioned earlier however, Guerrero-Preston and colleagues did show a negative correlation between cord serum PFOA concentrations and cord serum global methylation as determined by ELISA with marginal statistical significance ($p = 0.06$) (22). The mean concentrations of PFOS and PFOA in cord serum in that previous study were 5.8 ng/mL and 1.8 ng/mL respectively, which is similar to the exposure levels observed in our study. However, the different methods for determining global methylation on different target tissues may account for the different results within the two studies. Guerrero-Preston and colleagues (22) used ELISA to determine total methylated cytosine whereas our study measured *LINE1* as a surrogate marker for global methylation. In addition, they analyzed cord serum DNA methylation, which is associated with cell-free DNA circulating in the blood, whereas we analyzed cord blood methylation, which includes leucocyte DNA. Finally, their study

population contained only 15 pairs of newborns from smoking and non–smoking mothers, and owing to this small sample size, the authors could adjust for only a single variable, either maternal age or gestational age, and may not have adequately controlled for other potential confounding factors. Future epidemiological studies with larger sample sizes will clarify the effects of PFOA and other PFAAs *in utero* on offspring global DNA methylation.

Various factors, including oxidative stress and glutathione depletion, can influence DNA methylation (42). *In vitro* studies have shown that oxidative stress can be increased, and the levels of glutathione depleted, by exposure to PFAAs (43, 44). On the other hand, the epigenetic machinery itself, through DNA methyltransferase activity or histone modification, can cause changes in DNA methylation. PFAAs can act as ligands to activate peroxisome proliferator-activated receptors (PPARs) (45), whose targets include genes encoding histone-modifying enzymes (46). Although the exact mechanism-of-action has yet to be fully elucidated, it is possible that PFAAs modify DNA methylation through binding to PPARα and γ, and by influencing the epigenetic machinery directly. As is the case for PFAAs, phthalate metabolites such as mono-(2-ethylhexyl) phthalate (MEHP) can act as ligands for PPARs (47). LaRocca and colleagues reported a negative correlation between maternal urinary phthalate metabolites in early pregnancy and placental *IGF2* methylation from 179 placental samples taken from mothers enrolled in two birth cohort studies (48). That finding is in line with our observations on PFOA exposure and reduced *IGF2* methylation in cord blood,

which may be a consequence of PFOA and MEHP sharing a similar mode of action through PPARs.

A stronger association between PFOA exposure and *IGF2* methylation was observed than for PFOS. Previous studies have shown that PFOA has higher placental transfer efficiency than PFOS does (49, 50). PFOA is also a stronger agonist of PPARs than is PFOS (51). Both of these factors may explain the stronger association of PFOA with *IGF2* methylation.

We also found stronger associations of prenatal PFOS exposure with ponderal index than for PFOA exposure yet found only a weak association between PFOS and reduced *IGF2* methylation. Conversely, we observed stronger associations between PFOA and reduced *IGF2* methylation, which mediated some of the association with lower ponderal index. We have previously reported that prenatal PFOS exposure resulted in reduced birth weight among female infants (9). Our recent studies have also indicated that prenatal PFOS exposure results in greater physiological changes than PFOA exposure such as reduced fatty acids levels in pregnant women (10), and thyroid (11) or reproductive hormone imbalance (12) in infants and mothers. Due to these physiological changes during pregnancy, prenatal PFOS exposure may exert a greater effect on birth size when compared with PFOA.

On the other hand, it is possible that PFOA exposure may exert more long-term effects rather than effects observed at birth. We recently reported that prenatal exposure to

PFOA, but not PFOS, had negative associations with the mental developmental index scores of the Bayley Scales of Infant Development second edition (BSID II) among 6-month-old female infants (32). In a Danish birth cohort study, Halldorsson and colleagues reported that PFOA exposure *in utero* resulted in obesogenic phenotypes and excessive weight gain among 20-year-old females (52). These results suggest a long-term effect of PFOA, rather than an effect at birth, and the possibility that DNA methylation might mediate such long-term outcomes. However, we may not have been able to detect subtle effects of PFOS on *IGF2* methylation owing to our small sample size; further studies should be conducted to evaluate our findings.

The strength of our study is that it is a birth cohort study that has followed participants from the prenatal period assessing PFAA exposure in maternal blood during pregnancy and DNA methylation in cord blood at birth, which allows interpretations of causal relationships with chronological associations between exposures and outcomes. In addition, measurements of DNA methylation were done by pyrosequencing, which is a high-resolution method to determine site-specific methylation.

We also acknowledge that there are some limitations in our study. First, our birth cohort study is based on a single obstetrics and gynecology hospital. However, the hospital treated the largest number of deliveries in Sapporo city at the time, and when compared to another birth cohort study that covered 37 hospitals in the Hokkaido prefecture, our cohort

did not show any notable differences in the characteristics of mothers and infants (24). Of the pregnant women we initially approached (N=1796), 29% agreed to participate in the birth cohort (N=514). It is possible that some form of self-selection bias may have been introduced at this point. However, as Nilsen and colleagues have suggested, based on comparison of the data from the Norwegian Mother Child Cohort Study (MoBa) with that of the national medical birth registry, a self-selection bias in exposure-outcome association is likely to be small in prospective study designs (53). On the other hand, among our original birth cohort (N=514), the current study population (N=177) was selected for those who have both maternal and cord blood samples, thus limiting the study to mothers who delivered vaginally. Compared to those not included in the study, our study population consisted of younger, as well as a greater number of primiparous, mothers, and infants with longer gestational age, larger birth weight, and length (Table S4), suggesting that the current study population consisted of healthier participants, and may not be a representative of the source population. However, the aim of our study was to evaluate exposure-outcome association. In this context, the associations we observed could be applicable to other populations with a similar exposure range. In fact, it is possible that we underestimated the effect of PFAAs on birth size because the study population comprises healthier participants than those who excluded and had smaller birth size that could potentially be a result of prenatal PFAA exposure (7).

Secondly, the maternal blood sampling period for PFAA measurements differed

between subjects. Due to an increment of blood volume during pregnancy, PFAAs concentrations can appear 'diluted' as pregnancy progresses even if inherent exposure levels are the same, thus reduce the comparability of PFAAs exposure levels between individuals. However, PFAAs have relatively long half-lives, and we adjusted for the blood sampling period in the multivariate models to minimize this effect on our results. Additionally, we excluded 58 subjects whose maternal blood was collected after birth. These samples were not suitable to assess PFAA exposure levels during pregnancy due to large amount of blood loss at delivery. Although this exclusion further limited sample size of our study, it enabled us to evaluate the effects of prenatal exposure to PFAAs more clearly by excluding potentially unsuitable samples. Thirdly, our study did not have a large enough sample size to maintain statistical power; it is possible therefore that some milder effects of PFAAs on DNA methylation or infant birth size may not have been detected.

In conclusion, we found that prenatal PFOA exposure resulted in reduced *IGF2* methylation in cord blood, which in turn was associated with reduced ponderal index at birth. Our mediation analysis suggested that the effects of PFOA on reduced poderal index were mediated by *IGF2* methylation by approximately 21% of the total effect. Our group previously reported that plasma PFOS and PFOA concentrations had been decreasing in Hokkaido, Japan throughout the 2003-2011 period, whereas concentrations of perfluorononanoic acid (PFNA) and perfluorodecanoic acid (PFDA), have been increasing

(54). *In vitro* studies have suggested that PFAAs with longer carbon chains, such as PFNA and PFDA, are more potent in activating PPARs and exhibit more cytotoxicity (55, 56). Therefore, it will also be necessary to evaluate the effects of these emerging PFAAs on the fetal epigenome. As Halldorsson and colleagues have indicated, the effect of prenatal PFOA exposure may permanently modify health aspects of offspring including obesity and being overweight even twenty years later (52). Our result suggests the involvement of DNA methylation in affecting offspring growth at birth upon prenatal PFOA exposure. Following the participants of our birth cohort who were exposed to PFAAs including PFOA, and exhibited lower *IGF2* methylation will elucidate the long-term health effects associated with prenatal PFAAs exposure.

Conflict of Interest Statement

The authors declare they have no actual or potential competing financial interests.

Acknowledgments

We thank the staff at Sapporo Toho Hospital and the participating mothers and infants for their collaboration. We also thank Y. M. Ito for advice on statistical analyses, T. A. Yila for

advice on epigenetics, and R. Ito and Y. Iwasaki for PFAAs measurements. This study was supported by a Grant-in-Aid for Health Science Research from the Japanese Ministry of Health, Labour and Welfare (No. 717L00204); and by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology (No. 23390157, 25253050). We also would like to thank Editage (www.editage.jp) for English language editing.

Supplementary information is available at Journal of Exposure Science and Environmental Epidemiology's website.

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Figure legends

Figure 1. Dose-dependent relationships between quartiles in PFAAs and *IGF2* methylation (N=177)

Squared dots indicate beta, error bars depict 95%CI. *IGF2* methylation began to decline at third quartile (>1.4 ng/mL). Only PFOA showed a significant decreasing trend (p for trend = 0.007).

PFOS (ng/mL): Q1 (≤3.85), Q2 (3.85-5.30), Q3 (5.30-7.15), Q4 (>7.15); PFOA (ng/mL): Q1 (≤0.9), Q2 (0.9-1.4), Q3 (1.4-2.1), Q4 (>2.1)

Adjusted for maternal age, maternal education, maternal smoking during pregnancy, infant sex, maternal blood sampling period.

Figure 2. Mediation analysis of the association between PFOA and *IGF2* methylation and between PFOA and ponderal index (N=175)

Regression coefficients of each path are described alongside with arrows. The total effect of PFOA on ponderal index is described within parentheses.

A significant indirect effect of PFOA on ponderal index through *IGF2* hypomethylation was observed (*ab* = -0.11, BCa CI [-0.30, -0.02]). The *IGF2* methylation as a mediator can account for about 21% of the total effect (P_M = 0.21).

Adjusted for maternal age, pre-pregnancy BMI, parity, maternal education, maternal smoking during pregnancy, gestational age, infant sex, maternal blood sampling period.

ab: indirect effect, BCa CI: bias-corrected and accelerated confidence interval, P_M: percent mediation

* $p < 0.05$, ** $p < 0.01$

Table 1. Concentrations of PFOS and PFOA (ng/mL) in maternal serum during pregnancy (N = 177).

	Detection limit	Detection Rate (%)†	Mean (±SD)	Geometric Mean	Min	Percentile 25th	50th	75th	Max
PFOS	0.5	100.0	5.7 (±2.7)	5.2	1.5	3.9	5.3	7.2	16.2
PFOA	0.5	94.4	1.6 (±0.9)	1.3	ND	0.9	1.4	2.1	5.3

† For subjects with a level below the detection limit, we used a value equal to half the detection limit.

Table 2. Maternal and infant characteristics and their relationships with maternal serum PFAAs concentrations (N=177).

		PFOS (ng/ml)		PFOA (ng/ml)	
	Mean ±SD N (%)	ρ Median (25th-75th)	p	ρ Median (25th-75th)	p
Maternal characteristics					
Maternal age (year)[a]	29.8 ±4.8	ρ = -0.105	0.165	ρ = -0.067	0.376
Pre-pregnancy BMI (kg/m^2)[a]	21.2 ±3.1†	ρ = -0.019	0.799	ρ = -0.08	0.292
Parity[b]					
0	98 (55.4)	6.0 (4.2, 8.0)	0.008**	1.6 (1.2, 2.4)	<0.001**
1	59 (33.3)	4.8 (3.1, 6.2)		1.1 (0.8, 1.4)	
≥ 2	20 (11.3)	4.6 (3.3, 6.6)		0.8 (0.5, 1.1)	
Educational level (year)[b]					
≤ 12	83 (46.9)	5.2 (4.0, 7.0)	0.846	1.2 (0.8, 1.8)	0.030*
> 12	94 (53.1)	5.5 (3.7, 7.5)		1.5 (1.0, 2.3)	
Smoking during pregnancy[b]					
No	144 (81.4)	5.3 (4.0, 7.2)	0.308	1.4 (0.9, 2.2)	0.133
Yes	33 (18.6)	4.8 (2.8, 7.3)		1.2 (0.8, 1.7)	
Alcohol consumption during pregnancy[b]					
No	120 (67.8)	5.2 (3.9, 7.3)	0.891	1.4 (0.9, 2.1)	0.907
Yes	57 (32.2)	5.5 (3.7, 7.2)		1.4 (0.9, 2.2)	
Blood sampling period[c]					
23–31 weeks	67 (37.9)	6.0 (4.8, 7.6)	<0.001**	1.6 (1.0, 2.3)	0.015*
32-34 weeks	45 (25.4)	6.2 (3.9, 8.6)		1.4 (0.8, 1.9)	
35-41 weeks	65 (36.7)	4.6 (2.9, 5.9)		1.1 (0.8, 1.8)	
Infant characteristics					
Gestational age (week)[a]	39.8 ±1.0	ρ = 0.042	0.575	ρ = 0.063	0.403
Sex[b]					
Male	79 (44.6)	5.4 (4.2, 7.5)	0.158	1.6 (1.0, 2.2)	0.040*
Female	98 (55.4)	5.2 (3.3, 7.0)		1.3 (0.8, 1.9)	

Birth weight (g)[a]	3125 ±330	ρ = -0.126	0.096	ρ = -0.116	0.124
Birth length (cm)[a]	48.5 ±1.5‡	ρ = 0.091	0.232	ρ = 0.071	0.346
Ponderal Index (kg/m^3)[a]	27.4 ±2.2‡	ρ = -0.264	<0.001**	ρ = -0.226	0.003**

† Data missing: Pre-pregnancy BMI (N=1)

‡ Data excluded: Birth length (N=1), Ponderal Index (N=1)

[a] Spearman's correlation (ρ), [b] Mann-Whitney U-test, [c] Kruskal-Wallis

*p < 0.05, **p < 0.01

Table 3. Association between maternal PFAA concentrations and cord blood DNA methylation in multiple linear regression analyses (N=177).

	IGF2 methylation (%)		*H19* methylation (%)		*LINE1* methylation (%)	
	β (95% CI)	**p**	**β (95% CI)**	**p**	**β (95% CI)**	**p**
ln(PFOS)						
Crude	-0.60 (-1.53, 0.33)	0.203	-0.16 (-0.79, 0.46)	0.605	0.18 (-0.17, 0.53)	0.321
Adjusted	-0.56 (-1.56, 0.44)	0.274	-0.09 (-0.77, 0.59)	0.798	0.05 (-0.31, 0.42)	0.764
	R^2 = 0.056		*R^2 = 0.018*		*R^2 = 0.134*	
ln(PFOA)						
Crude	-0.82 (-1.50, -0.14)	0.019*	-0.10 (-0.56, 0.36)	0.673	-0.02 (-0.28, 0.24)	0.863
Adjusted	-0.73 (-1.44, -0.02)	0.043*	-0.08 (-0.57, 0.40)	0.736	-0.15 (-0.41, 0.10)	0.244
	R^2 = 0.072		*R^2 = 0.018*		*R^2 = 0.140*	

Adjusted for maternal age, maternal education, maternal smoking during pregnancy, infant sex, maternal blood sampling period.

β: partial regression coefficient and indicates methylation changes with ln-unit increase in concentration, *p < 0.05

R^2: squared multiple correlation coefficient in adjusted model.

Table 4. Association between DNA methylation and birth size in multiple linear regression analyses.

	Birth weight (g) (N=177)		Birth length (cm) (N=176)		Ponderal index (kg/m^3) (N=176)	
	β (95% CI)	**p**	**β (95% CI)**	**p**	**β (95% CI)**	**p**
***IGF2* methylation (%)**						
Adjusted for gestational age	11.5 (-3.8, 26.9)	0.140	-0.05 (-0.12, 0.02)	0.190	0.19 (0.08, 0.29)	0.001**
Full adjustment†	13.2 (-2.5, 28.8)	0.099	-0.03 (-0.10, 0.05)	0.443	0.17 (0.06, 0.27)	0.002**
	R^2 = 0.173		*R^2 = 0.15*		*R^2 = 0.166*	
***H19* methylation (%)**						
Adjusted for gestational age	-0.2 (-23.3, 23.0)	0.987	0.03 (-0.08, 0.14)	0.545	-0.06 (-0.22, 0.10)	0.470
Full adjustment†	7.9 (-16.1, 31.9)	0.515	0.05 (-0.06, 0.16)	0.362	-0.02 (-0.18, 0.15)	0.839
	R^2 = 0.162		*R^2 = 0.151*		*R^2 = 0.118*	
***LINE1* methylation (%)**						
Adjusted for gestational age	10.0 (-31.3, 51.3)	0.634	0.15 (-0.05, 0.34)	0.142	-0.15 (-0.44, 0.14)	0.306
Full adjustment†	-3.1 (-47.1, 40.9)	0.889	0.01 (-0.20, 0.21)	0.940	-0.04 (-0.34, 0.27)	0.807
	R^2 = 0.160		*R^2 = 0.147*		*R^2 = 0.119*	

† Data missing: pre-pregnancy BMI (N=1).

Full adjustment: adjusted for maternal age, pre-pregnancy BMI, parity, maternal education, maternal smoking during pregnancy, gestational age, infant sex.

β: partial regression coefficient and indicates methylation changes with ln-unit increase in concentration, **p < 0.01

R^2: squared multiple correlation coefficient in full-adjusted model.

Table 5. Association between maternal PFAA concentrations and infant birth size in multiple linear regression analyses.

	Birth weight (g) (N=177)		Birth length (cm) (N=176)		Ponderal index (kg/m^3) (N=176)	
	β (95% CI)	**p**	**β (95% CI)**	**p**	**β (95% CI)**	**p**
ln(PFOS)						
Adjusted for gestational age	-79.7 (-175.9, 16.6)	0.104	0.26 (-0.20, 0.72)	0.271	-1.19 (-1.85, -0.53)	0.001**
Full adjustment†	-56.0 (-162.8, 50.8)	0.302	0.32 (-0.19, 0.82)	0.216	-1.07 (-1.79, -0.36)	0.004**
	R^2 = 0.178		*R^2 = 0.159*		*R^2 = 0.187*	
ln(PFOA)						
Adjusted for gestational age	-62.4 (-133.7, 8.9)	0.086	0.07 (-0.27, 0.41)	0.689	-0.67 (-1.16, -0.17)	0.008**
Full adjustment†	-49.4 (-130.4, 31.6)	0.230	0.01 (-0.37, 0.40)	0.955	-0.44 (-0.99, 0.12)	0.123
	R^2 = 0.180		*R^2 = 0.151*		*R^2 = 0.156*	

† Data missing: pre-pregnancy BMI (N=1).

Full adjustment: maternal age, pre-pregnancy BMI, parity, maternal education, maternal smoking during pregnancy, gestational age, infant sex, maternal blood sampling period.

β: partial regression coefficient and indicates methylation changes with ln-unit increase in concentration, **p < 0.01

R^2: squared multiple correlation coefficient in full-adjusted model.

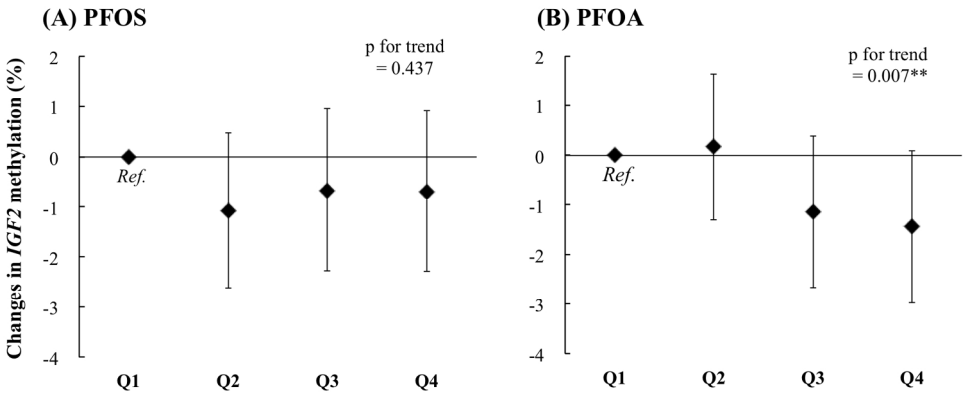

Figure 1. Dose-dependent relationships between quartiles in PFAAs and *IGF2* methylation (N=177). Squared dots indicate beta, error bars depict 95%CI. *IGF2* methylation began to decline at third quartile (>1.4 ng/mL). Only PFOA showed a significant decreasing trend (*p* for trend = 0.007).
PFOS (ng/mL): Q1 (≤3.85), Q2 (3.85-5.30), Q3 (5.30-7.15), Q4 (>7.15); PFOA (ng/mL): Q1 (≤0.9), Q2 (0.9-1.4), Q3 (1.4-2.1), Q4 (>2.1).
Adjusted for maternal age, maternal education, maternal smoking during pregnancy, infant sex, maternal blood sampling period.

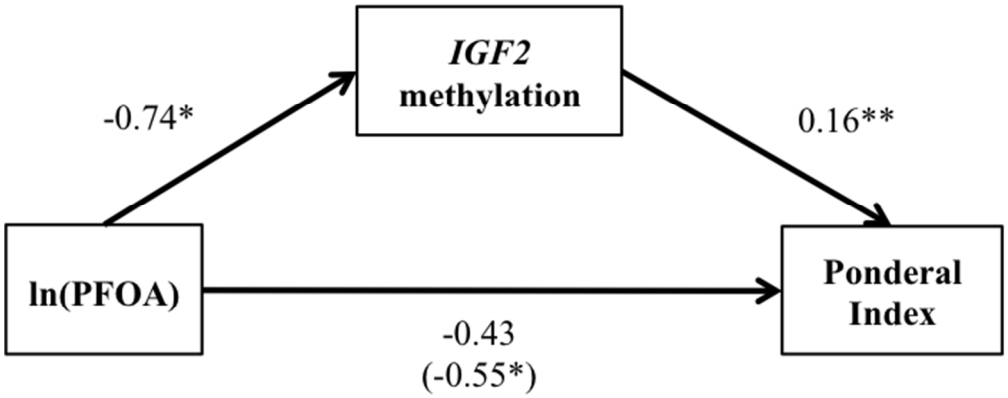

Figure 2. Mediation analysis of the association between PFOA and *IGF2* methylation and between PFOA and ponderal index (N=175).
Regression coefficients of each path are described alongside with arrows. The total effect of PFOA on ponderal index is described within parentheses.
A significant indirect effect of PFOA on ponderal index through *IGF2* hypomethylation was observed (*ab* = -0.11, BCa CI [-0.30, -0.02]). The *IGF2* methylation as a mediator can account for about 21% of the total effect (P_M = 0.21).
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ab: indirect effect, BCa CI: bias-corrected and accelerated confidence interval, P_M: percent mediation
* $p < 0.05$, ** $p < 0.01$

Supplementary Information

Contents

Table S1. Primers and PCR conditions for quantification in methylation at IGF2/H19 and LINE1

Name	Region†	PCR volume	DNA Template	Primers	Primer concentration	$MgCl_2$	PCR conditions	Sequence primers	Seq. primer concentration
IGF2 **DMR0**	chr11p15.5, site 1: 2,109,519; site 2: 2,109,516	10 µl	8.3 ng	**F:** 5'-GGA GGG GGT TTA TTT TTT TAG GAA G-3' **R:** 5'-[Biotin]-AAC CCC AAC AAA AAC CAC TAA ACA C-3'	0.2 µM	1.5 mM	95°C for 15 m; (94°C for 30s, 68°C for 30s, 72°C for 30s) x5; (94°C for 30s, 66°C for 30s, 72°C for 30s) x50; 72°C for 10 m	5'-GGG GTT TAT TTT TTT AGG A-3'	0.3 µM
H19 **DMR** ‡	chr11p15.5, site 1: 1,964,261; site 2: 1,964,259; site 3: 1,964,257; site 4: 1,964,254	38 µl	30.4 ng	**F:** 5'-TTT GTT GAT TTT ATT AAG GGA G-3' **R:** 5'-[Biotin]-CTA TAA ATA AAC CCC AAC CAA AC-3'	0.2 µM	3.0 mM	95°C for 15 m; (94°C for 30s, 65°C for 30s, 72°C for 30s) x5; (94°C for 30s, 62°C for 30s, 72°C for 30s) x5; (94°C for 30s, 59°C for 30s, 72°C for 30s) x50; 72°C for 10 m	5'-GTG TGG AAT TAG AAG T-3'	0.3 µM
		10 µl	8.3 ng						
LINE-1	—	38 µl	30.4 ng	**F:** 5'-TTT TGA GTT AGG TGT GGG ATA TA-3' **R:** 5'-[Biotin]-AAA ATC AAA AAA TTC CCT TTC-3'	0.2 µM	1.5 mM	95°C for 15 m; (95 °C for 30s, 53°C for 30s, 72°C for 30s) x35; 72°C for 7 m	5'-AGT TAG GTG TGG GAT ATA GT-3'	0.3 µM

† NCBI Human Genome Build 37.1

‡ Measurements at H19 DMR were performed in duplicate with two different reaction volumes (38 and 10 µl, respectively) in the same condition for the rest.

Table S2. Relationships between DNA methylation and maternal or infant characteristics (N=177).

		IGF2 (%)		*H19* (%)		*LINE1* (%)	
	N	ρ Median (25th-75th)	p	ρ Median (25th-75th)	p	ρ Median (25th-75th)	p
DNA methylation (Mean ±SD)	177	48.5 ±3.0		52.3 ±2.0		75.8 ±1.1	
Maternal characteristics							
Maternal age (year)[a]	177	ρ = 0.148	0.050*	ρ = 0.146	0.053	ρ = 0.146	0.053
Pre-pregnancy BMI (kg/m^2)[a]	176[†]	ρ = 0.085	0.26	ρ = -0.081	0.287	ρ = -0.059	0.439
Parity[b]							
0	98	48.5 (46.1, 50.2)	0.191	52.1 (51.0, 53.5)	0.929	75.8 (75.0, 76.4)	0.430
1	59	49.3 (46.7, 51.1)		52.0 (51.1, 53.5)		76.0 (75.4, 76.7)	
≥ 2	20	48.2 (46.9, 50.3)		52.1 (50.1, 53.8)		76.0 (75.0, 76.7)	
Educational level (year)[b]							
≤ 12	83	49.0 (46.4, 50.6)	0.657	52.0 (51.0, 53.4)	0.526	75.7 (75.0, 76.4)	0.077
> 12	94	48.6 (46.4, 50.2)		52.1 (50.9, 53.9)		76.1 (75.2, 76.6)	
Smoking during pregnancy[b]							
No	144	48.8 (46.4, 50.4)	0.738	51.9 (51.0, 53.4)	0.301	75.9 (75.1, 76.5)	0.679
Yes	33	48.9 (45.8, 50.5)		52.3 (51.0, 54.5)		75.7 (75.3, 76.4)	
Alcohol consumption during pregnancy[b]							
No	120	49.0 (46.7, 50.8)	0.124	52.1 (51.0, 53.5)	0.957	75.8 (75.2, 76.4)	0.187
Yes	57	47.9 (45.8, 50.1)		52.0 (51.0, 53.7)		76.1 (75.1, 76.6)	
Blood sampling period[c]							
23–31 weeks	67	48.9 (46.4, 50.2)	0.814	52.2 (51.0, 53.8)	0.895	76.1 (75.1, 76.7)	0.050
32-34 weeks	45	49.2 (46.6, 50.6)		51.9 (50.9, 53.5)		76.1 (75.6, 76.5)	
35-41 weeks	65	48.6 (46.2, 51.1)		52.0 (51.1, 53.5)		75.7 (75.0, 76.2)	
Infant characteristics							
Gestational age (week)[a]	177	ρ = 0.057	0.451	ρ = 0.02	0.794	ρ = 0.066	0.383
Sex[b]							
Male	79	48.7 (45.9, 50.0)	0.085	52.1 (51.0, 53.3)	0.808	76.3 (75.7, 76.9)	<0.001**

Female	98	49.0 (46.7, 51.0)		52.0 (51.0, 53.7)		75.6 (74.8, 76.1)	
Birth weight (g)[a]	177	ρ = 0.112	0.139	ρ = 0.043	0.574	ρ = 0.055	0.472
Birth length (cm)[a]	176‡	ρ = -0.079	0.300	ρ = 0.066	0.386	ρ = 0.138	0.068
Ponderal Index (kg/m^3)[a]	176‡	ρ = 0.222	0.003**	ρ = -0.036	0.640	ρ = -0.110	0.148

† Data missing: Pre-pregnancy BMI (N=1)

‡ Data excluded: Birth length (N=1), Ponderal Index (N=1)

[a] Spearman's correlation (ρ), [b] Mann-Whitney U-test, [c] Kruskal-Wallis

*p < 0.05, **p < 0.01

Table S3. Effects of PFAAs on *IGF2* methylation in quartiles (N=177).

	PFOS (log_{10})				PFOA (log_{10})			
	N	**(ng/mL)**	**β (95% CI)**	**p**	**N**	**(ng/mL)**	**β (95% CI)**	**p**
1st Quartile	44	≤ 3.85	*Ref.*		53	≤ 0.9	*Ref.*	
2nd Quartile	47	3.85-5.30	-1.07 (-2.62, 0.48)	0.244	43	0.9-1.4	0.17 (-1.30, 1.64)	0.986
3rd Quartile	42	5.30-7.15	-0.66 (-2.27, 0.95)	0.640	40	1.4-2.1	-1.14 (-2.66, 0.38)	0.191
4th Quartile	44	> 7.15	-0.69 (-2.29, 0.9)	0.598	41	> 2.1	-1.44 (-2.98, 0.1)	0.074
p for trend			*p = 0.437*				*p = 0.007***	

Adjusted for maternal age, maternal education, infant sex, smoking during pregnancy, blood sampling period.

**p < 0.01

Table S4. Comparison of characteristics in Toho cohort participants between those included and not included in the final analysis.

	Final analysis (N=177)		Not included (N=337)		
		Median (25-75th)		Median (25-75th)	
	N	Percent	N	Percent	p
PFOS (ng/mL)[a]	177	5.3 (3.9, 7.2)	270	5.2 (3.3, 7.0)	0.206
PFOA (ng/mL)[a]	177	1.4 (0.9, 2.1)	270	1.2 (0.8, 1.6)	0.007**
Maternal characteristics					
Maternal age (year)[a]	177	30 (27, 33)	333	30 (28, 34)	0.048*
Pre-pregnancy BMI (kg/m^2)[a]	176	20.6 (19.4, 22.0)	329	20.5 (19.1, 22.4)	0.680
Parity[b]					
0	98	(55.4 %)	142	(43.6 %)	0.039*
1	59	(33.3 %)	135	(41.4 %)	
≥ 2	20	(11.3 %)	49	(15.0 %)	
Educational level (year)[b]					
≤ 12	83	(46.9 %)	148	(43.9 %)	0.520
> 12	94	(53.1 %)	189	(56.1 %)	
Smoking during pregnancy[b]					
No	144	(81.4 %)	261	(78.9 %)	0.502
Yes	33	(18.6 %)	70	(21.1 %)	
Alcohol consumption during pregnancy[b]					
No	120	(67.8 %)	237	(70.3 %)	0.555
Yes	57	(32.2 %)	100	(29.7 %)	
Blood sampling period[b]					
23–31 weeks	67	(37.9 %)	86	(26.7 %)	<0.001**
32-34 weeks	45	(25.4 %)	50	(15.5 %)	
35-41 weeks	65	(36.7 %)	38	(11.8 %)	
After birth	0	(0.0 %)	148	(46.0 %)	
Infant characteristics					
Gestational age (week)[a]	177	40.0 (39.3, 40.6)	327	39.1 (38.0, 40.1)	<0.001**
Sex[b]					
Male	79	(44.6 %)	163	(49.8 %)	0.263
Female	98	(55.4 %)	164	(50.2 %)	

Caesarean section[b]					
No	177	(100.0 %)	220	(67.3 %)	<0.001**
Yes	0	(0.0 %)	107	(32.7 %)	
Birth weight (g)[a]	177	3098 (2926, 3321)	327	3006 (2756, 3270)	<0.001**
Birth length (cm)[a]	177	48.4 (47.5, 49.4)	327	47.8 (46.5, 49.0)	<0.001**
Ponderal Index (kg/m^3)[a]	177	27.4 (25.7, 28.9)	327	27.3 (25.8, 28.8)	0.832

[a] Mann-Whitney U-test, [b] Chi-square test

*p < 0.05, **p < 0.01

Fig S1. Flow chart of study participant selection.