Title	Effects of prenatal perfluoroalkyl acid exposure on cord blood IGF2/H19 methylation and ponderal index : The Hokkaido Study
Author(s)	Kobayashi, Sachiko; Azumi, Kaoru; Goudarzi, Houman; Araki, Atsuko; Miyashita, Chihiro; Kobayashi, Sumitaka; Itoh, Sachiko; Sasaki, Seiko; Ishizuka, Mayumi; Nakazawa, Hiroyuki; Ikeno, Tamiko; Kishi, Reiko
Citation	Journal of Exposure Science and Environmental Epidemiology, 27(3), 251-259 https://doi.org/10.1038/jes.2016.50
Issue Date	2017-05
Doc URL	http://hdl.handle.net/2115/65204
Type	article (author version)
File Information	Sa_Kobayashi_JESEE.pdf

[Instructions for use](#)

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

Sachiko Kobayashi (Bachelor's)¹, Kaoru Azumi (PhD)¹, Houman Goudarzi (PhD)¹, Atsuko
Araki (PhD)¹, Chihiro Miyashita (PhD)¹, Sumitaka Kobayashi (PhD)¹, Sachiko Itoh (PhD)¹,
Seiko Sasaki (PhD)², Mayumi Ishizuka (PhD)³, Hiroyuki Nakazawa (PhD)⁴, Tamiko Ikeno
(PhD)¹, Reiko Kishi (PhD)^{1*}

¹Hokkaido University Center for Environmental and Health Sciences, Sapporo, Japan

²Hokkaido University Graduate School of Medicine, Department of Public Health Sciences,
Sapporo, Japan

³Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate
School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

⁴Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University,
Tokyo, Japan

Correspondence: Reiko Kishi, MD, PhD, MPH, Eminent Professor, Center for Environmental
and Health Sciences, Hokkaido University, North 12 West 7, Kita-ku, Sapporo, 060-0812,

1
2
3
4 18 Japan. Tel.: +81-11-706-4746. Fax: +81-11-706-4725. E-mail: rkishi@med.hokudai.ac.jp
5
6

7 19
8
9

10 20 **Running title.** Prenatal PFAAs exposure and *IGF2/H19* methylation
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4 21 **Abstract**
5

6
7 22 Prenatal exposure to perfluoroalkyl acids (PFAAs) influences fetal growth and
8
9
10 23 long-term health. However, whether PFAAs affect offspring DNA methylation patterns to
11
12 24 influence health outcomes is yet to be evaluated. Here, we assessed effect of prenatal PFAA
13
14
15 25 exposure on cord blood *insulin-like growth factor 2 (IGF2)*, *H19*, and *LINE1* methylation and
16
17
18 26 its associations with birth size. Mother-child pairs (N = 177) from the Hokkaido Study on
19
20
21 27 Environment and Children's Health were included in the study. Perfluorooctane sulfonate
22
23
24 28 (PFOS) and perfluorooctanoic acid (PFOA) levels in maternal serum were measured by
25
26
27 29 liquid chromatography-tandem mass spectrometry. *IGF2*, *H19*, and *LINE1* methylation in
28
29
30 30 cord blood DNA was determined by pyrosequencing. After full adjustment in multiple linear
31
32
33 31 regression models, *IGF2* methylation showed a significant negative association with ln-unit
34
35
36 32 increase in PFOA (partial regression coefficient = -0.73, 95% confidence interval: -1.44 to
37
38
39 33 -0.02). Mediation analysis suggested that reduced *IGF2* methylation explained approximately
40
41
42 34 21% of the observed association between PFOA exposure and reduced ponderal index of the
43
44
45 35 infant at birth. These results indicated that effects of prenatal PFOA exposure could be
46
47
48 36 mediated through DNA methylation. Further study will be required to determine the potential
49
50
51 37 for long-term adverse health effects of reduced *IGF2* methylation induced by PFOA
52
53
54 38 exposure.
55

56 39
57
58
59
60

1
2
3
4 40 **Key words:** epidemiology; perfluorinated chemicals; child exposure/health; endocrine

5
6
7 41 disruptors; DNA methylation; birth cohort

8
9
10 42

11
12
13 43

44 **Introduction**

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

56 Earlier epidemiological studies have reported reductions in birth size associated with
57 prenatal PFAAs exposure (7, 8). We have reported, for example, a 269.4 g reduction in birth
58 weight associated with a log₁₀-unit increase of maternal PFOS concentrations among female
59 infants (9). In the same cohort from that study, we observed that maternal PFOS
60 concentrations during pregnancy are negatively associated with triglyceride and essential
61 fatty acids levels in the pregnant women (10). We also demonstrated that prenatal exposure to
62 PFAAs could result in disruption of thyroid (11) and reproductive hormone balance (12) in

1
2
3
4 63 infants and mothers. Thus, one explanation for the effects of PFAAs on birth size is induction
5
6
7 64 of maternal and fetal physiological changes, including altered metabolic or hormone
8
9
10 65 endpoints.

11
12 66 Another possible explanation is that prenatal PFAAs exposure modifies the
13
14
15
16 67 epigenetic machinery of developing fetuses leading to changes in gene expression and
17
18
19 68 subsequent phenotypes. DNA methylation is an epigenetic process that plays a role in
20
21
22 69 embryonic development and cellular differentiation. It occurs by addition of a methyl group
23
24
25 70 to a cytosine at cytosine–guanine dinucleotide (CpG) loci and acts like a switch on gene
26
27
28 71 expression (13). This DNA methylation is heritable through cell division but can also be
29
30
31 72 affected by the external environment and has therefore been postulated as a mediator of
32
33
34 73 intrauterine environmental influences on postnatal phenotypes (14).

35
36 74 Previous epidemiological studies have suggested an association between some
37
38
39 75 prenatal environments and cord blood DNA methylation at repetitive sequences such as the
40
41
42 76 long interspersed element 1 (*LINE1*) (15). *LINE1* is globally distributed comprising
43
44
45 77 approximately 17% of the human genome. Methylation of *LINE1* is correlated with DNA
46
47
48 78 methylation across the entire genome and can be used as a surrogate for global DNA
49
50
51 79 methylation (16). The insulin–like growth factor 2 (*IGF2*)/*H19* locus is also one of the most
52
53
54 80 studied regions in epigenetics. IGF2 is a peptide hormone essential for fetal growth and is
55
56
57 81 expressed from early embryonic stages throughout fetal development. Expression of this
58
59
60

1
2
3
4 82 imprinted gene is controlled by DNA methylation at the *IGF2* differentially methylated
5
6
7 83 region (DMR) and *H19* DMR, which are reciprocally methylated and expressed between
8
9
10 84 maternal and paternal alleles (17). It has been suggested that, in addition to its biological
11
12
13 85 function, *IGF2* methylation may serve as an epigenetic marker for intrauterine disruption.
14
15
16 86 Through epidemiological studies, reduced *IGF2* methylation in cord blood or placental
17
18
19 87 tissues has been correlated with reduced fetal growth features such as lower birth weight and
20
21
22 88 small-for-gestational age (SGA) (18, 19). Emerging evidence has indicated a role for the
23
24
25 89 *IGF2* gene in metabolic disorders such as obesity and hypertension in humans (20). One
26
27
28 90 report has suggested that *IGF2/H19* methylation at birth is linked to the development of
29
30
31 91 obesity in early childhood (21).

32
33 92 Whether levels of PFAAs can influence infant health effects through an epigenetic
34
35
36 93 mechanism has not been rigorously evaluated in epidemiological studies. In an earlier study,
37
38
39 94 Guerrero-Preston and colleagues observed that cord blood PFOA concentrations negatively
40
41
42 95 correlated with cord serum global DNA methylation (22). However, this study was
43
44
45 96 cross-sectional with a limited sample size (N=30) and could not control for multiple potential
46
47
48 97 confounders. Furthermore, given that the epigenetic machinery may work in a site- and
49
50
51 98 gene-specific manner, the effects of PFAAs on gene-specific as well as global DNA
52
53
54 99 methylation need to be evaluated. These evaluation of the effects of PFAAs on both global
55
56
57 100 and gene-specific methylation and their association with health outcomes at birth based also
58
59
60

1
2
3
4 101 necessitate larger sample sizes than the above earlier investigation.
5
6

7 102 We hypothesized that prenatal exposure to PFAAs modifies infant DNA methylation
8
9
10 103 in genes essential for fetal growth, which in turn contributes to reduced birth size. In this
11
12 104 study, we evaluated the effects of prenatal PFOS and PFOA exposure on gene-specific
13
14
15 105 *IGF2/H19* methylation as well as *LINE1* methylation (as a surrogate for global DNA
16
17
18 106 methylation), and their association with birth weight, length and ponderal index.
19
20

21 107
22
23

24 108 **Materials and Methods**

25
26

27 109 ***Study population.*** Study participants were enrolled as a part of the Hokkaido Study
28
29
30 110 on Environment and Children's Health, an ongoing birth cohort study. Details of the study
31
32 111 design were described previously (23, 24). Briefly, pregnant women at 23-35 weeks of
33
34
35 112 gestation were recruited at Toho Hospital, Sapporo, Japan between 2002 and 2005. Of 1796
36
37
38 113 pregnant women initially approached, the following were excluded from recruitment:
39
40
41 114 registrants of the Japanese cord blood bank (25 %) or women planning to deliver at another
42
43
44 115 hospital (3 %). Subsequently, 514 women agreed to participate in this study (29 %). Of those,
45
46
47 116 based on pre-established criteria, 10 were later excluded due to miscarriage or stillbirth (N =
48
49
50 117 2), relocation (N = 1), voluntary withdrawal (N = 7). Of 504 subjects whose birth records and
51
52
53 118 baseline questionnaires were available, we further excluded multiple births (N = 7).
54
55
56 119 Subsequently, a total of 441 maternal blood samples and 267 cord blood samples were
57
58
59
60

1
2
3
4 120 available for PFAA and DNA methylation analyses. Among subjects who could provide both
5
6
7 121 maternal and cord blood samples, we excluded mothers whose blood was obtained after
8
9
10 122 delivery (N=58). Overall, 177 mother-infant pairs were evaluated in this study (Fig S1).

11
12
13 123 The study was conducted after written informed consent was received from all
14
15
16 124 subjects. The institutional ethical board for human gene and genome studies at Hokkaido
17
18
19 125 University Center for Environmental and Health Science and Hokkaido University Graduate
20
21
22 126 School of Medicine approved the study protocol.

23
24 127 **Data collection.** At enrollment, a self-administered questionnaire was used to obtain
25
26
27 128 baseline information including parental demographic characteristics, anthropometric
28
29
30 129 measurements, and lifestyle factors such as maternal smoking and alcohol consumption (9).
31
32
33 130 Information on pregnancy complications, gestational age, infant gender, and birth weight and
34
35
36 131 birth length was obtained from medical records.

37
38
39 132 **Specimen collection.** A 40 mL aliquot of maternal blood samples was collected from
40
41
42 133 participants between 24-41 weeks of gestational age. Cord blood samples were taken
43
44
45 134 immediately after birth. All specimens were stored at -80°C prior to analysis.

46
47 135 **Exposure assessment.** Detailed methods for the measurement of PFOS and PFOA
48
49
50 136 have been previously described (5, 25). Briefly, serum samples (0.1 mL) were mixed with 0.2
51
52
53 137 mL internal standard solution containing acetonitrile, centrifuged at $1450 \times g$ for 10 min, and
54
55
56 138 the supernatant was then transferred to a polypropylene tube. An aliquot of the filtered

1
2
3
4 139 sample solution was subjected to column-switching liquid chromatography-tandem mass
5
6
7 140 spectrometry (LC–MS/MS). PFOS levels were detected in all samples. PFOA levels for 10
8
9
10 141 samples (5.6 %) were below the detection limit (0.50 ng/mL). The percentage of samples
11
12
13 142 below the detection limit was small enough to yield fair estimates with a simple substitution
14
15
16 143 approach (26, 27). Therefore, for samples below the detection limit, we assigned a value of
17
18
19 144 half the detection limit (0.25 ng/mL).

20
21 145 ***Quantification of DNA methylation.*** Genomic DNA was extracted from umbilical
22
23
24 146 cord blood using a Maxwell® 16 DNA Purification Kit (Promega, Madison, WI, USA). DNA
25
26
27 147 (500 ng) was subjected to a bisulfite conversion using Epitect Plus Bisulfite Kit (Qiagen,
28
29
30 148 Venlo, Netherlands) to convert unmethylated cytosine to uracil, leaving the methylated
31
32
33 149 cytosine unaltered. Bisulfite pyrosequence was performed as previously described (28, 29).
34
35
36 150 We evaluated two *IGF2/H19* regulatory regions; the first was two CpG sites comprising the
37
38
39 151 intragenic *IGF2* differentially methylated region 0 (DMR0), and the other was four CpG sites
40
41
42 152 within *H19* DMR. For *LINE1*, three CpG sites at the promoter region were quantified. Each
43
44
45 153 locus was amplified using HotStarTaq DNA polymerase in PyroMark PCR Kit (Qiagen).
46
47
48 154 Biotin-labeled single-stranded amplicons were bound to the Streptavidin Sepharose HP
49
50
51 155 (Amersham Biosciences, Uppsala, Sweden) and purified using the Pyromark Q24 Work
52
53
54 156 Station (Qiagen). Pyrosequencing was performed using Pyromark Q24 system and data was
55
56
57 157 analyzed using Pyro Q-CpG Software (Qiagen). PCR primers, conditions, and sequencing
58
59
60

1
2
3
4 158 primers are described in Table S1. All the investigators were blinded to the individual's
5
6
7 159 information related to the study at the time of the experiment. All samples were analyzed in
8
9
10 160 duplicate. Since methylation levels of each site in the given three regions were highly
11
12
13 161 correlated, we averaged the methylation levels of each region (*IGF2*, *H19*, and *LINE1*) and
14
15
16 162 used the average in subsequent analyses.

17
18 163 ***Data Analysis.*** The Spearman's correlation test, Mann-Whitney U-test, and
19
20
21 164 Kruskal–Wallis test were applied to determine whether maternal and offspring characteristics
22
23
24 165 were associated with PFAAs exposure. Multiple linear regression analyses were performed to
25
26
27 166 determine associations between maternal PFAA concentrations and infant methylation levels
28
29
30 167 at each locus. Because of skewed distributions, we transformed the PFOS and PFOA
31
32
33 168 concentrations in maternal serum to the natural log (ln) scale. Maternal blood sampling
34
35
36 169 period for PFAA measurement was categorized into three groups, 23-31 weeks, 32-34 weeks,
37
38
39 170 and 35-41 weeks of pregnancy. Multiple linear regression analyses were applied to estimate
40
41
42 171 the effects DNA methylation on infant birth weight, length, and ponderal index, which was
43
44
45 172 calculated as the ratio of birth weight (kg) to cubed birth length (m^3) (30). One subject was
46
47
48 173 excluded from the analysis of birth length and ponderal index *post hoc* because its birth
49
50
51 174 length departed from the mean by more than four standard deviations (SDs), despite its
52
53
54 175 gestational age and birth weight being within normal range. Covariates were selected based
55
56
57 176 on associations between dependent and independent variables observed in our data or
58
59
60

177 previous studies: in DNA methylation models, we adjusted for maternal age, maternal
 178 education, infant sex, maternal smoking during pregnancy, and blood sampling period; in
 179 birth size models, we adjusted for gestational age, maternal age, pre-pregnancy BMI, parity,
 180 maternal education, maternal smoking during pregnancy, and infant sex. We additionally
 181 implemented directed acyclic graphs (DAGs) using *DAGitty* ver. 2.3 (31) for covariate
 182 selection, and confirmed that the covariates selected above were neither colliders nor
 183 intermediates. As Bartlett's test verified the assumption of equal variance among groups, in
 184 quartile models, PFOS and PFOA concentrations were divided into quartiles and a test for a
 185 linear trend was performed by linear contrast. Adjusted least square means of methylation
 186 levels in each quartile were compared by the Hsu-Dunnett's method accounting for multiple
 187 comparisons. For calculation of p for trend, we used linear contrast coefficients -3, -1, +1, +3
 188 assigned to quartiles 1, 2, 3, and 4, respectively (10, 32). Mediation analysis was performed
 189 by PROCESS, a macro implemented in SPSS (33). It estimates how an effect of X
 190 (independent variable) can be apportioned into its indirect effect on Y (dependent variable)
 191 through M (mediator), and its direct effect on Y . The indirect effect of X on Y through M can
 192 be quantified as the product of two coefficients: a (the effect of X on M) and b (the effect of
 193 M on Y) (i.e. ab). The bias-corrected and accelerated confidence intervals (BCa CIs) of the
 194 indirect effect (ab) are calculated by bootstrapping with 1,000 iterations. The effect size of
 195 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 ($\pm$ SD) for *IGF2* DMR0, *H19* DMR and *LINE1* were 48.5% ($\pm$ 3.0), 52.3% ($\pm$ 2.0) and 75.8% ($\pm$ 1.1), respectively. Geometric means (GM) (25th to 75th 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 ($\beta = -0.44$, 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.

242

243 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 $\alpha = 0.05$ 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 PFAs 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

1
2
3
4 253 which PFAAs affect fetal growth still require elucidation. An original finding of this current
5
6
7 254 study is the involvement of *IGF2* methylation in the effect of prenatal PFOA exposure on
8
9
10 255 ponderal index at birth. To date, only a small number of epidemiological studies have
11
12
13 256 investigated the effects of PFAAs on DNA methylation, and these have focused on global
14
15
16 257 DNA methylation, rather than gene-specific methylation as in this study. Two studies on adult
17
18
19 258 populations have suggested the possibility of an epigenetic effect of PFAAs on global
20
21
22 259 methylation. In the C8 Health Project, Watkins and colleagues studied 685 adult participants
23
24
25 260 who lived in a PFAA-contaminated area. The authors found a significant monotonic increase
26
27
28 261 in *LINE1* methylation across PFOS and PFNA tertiles in the peripheral blood leucocytes (37).
29
30
31 262 Leter and colleagues studied 262 fertile men from three independent populations in
32
33
34 263 Greenland and Eastern Europe. When the three populations were analyzed independently, the
35
36
37 264 authors found a significant association between PFAAs and *LINE1* methylation as well as
38
39
40 265 total cytosine methylation in sperm DNA. However, when the data was analyzed across the
41
42
43 266 total combined population, no significant associations between PFAA exposure and DNA
44
45
46 267 methylation were observed (38).

47
48 268 Prior to this study, there was only one small cross-sectional study that had
49
50
51 269 evaluated the effect of prenatal PFAA exposure on offspring DNA methylation (22). This was
52
53
54 270 conducted by Guerrero-Preston and colleagues who investigated 15 pairs of newborns of
55
56
57 271 smoking mothers and mothers no history of smoking. They demonstrated that cord blood
58
59
60

1
2
3
4 272 PFOA concentration was negatively correlated with cord serum global methylated cytosine as
5
6
7 273 determined by enzyme-linked immuno-sorbent assay (ELISA).
8
9

10 274 As noted earlier, the above studies focused on global DNA methylation, rather than
11
12 275 gene-specific methylation and neither did they evaluate whether methylation changes induced
13
14
15 276 by PFAAs exposure could influence health outcomes such as birth size reduction. In this
16
17
18 277 context, it should be noted that the association between birth size and *IGF2/H19* methylation
19
20
21 278 has been relatively well studied. Previous epidemiological studies have reported that reduced
22
23
24 279 *IGF2* methylation in cord blood and placental tissues is correlated with reduced fetal growth
25
26
27 280 such as lower birth weight or small-for-gestational age (SGA) (18, 19). Our study, which to
28
29
30 281 our knowledge, is the first to report that prenatal PFOA exposure results in reduced cord
31
32
33 282 blood *IGF2* methylation, which in turn is associated with decreased ponderal index at birth, is
34
35
36 283 therefore consistent with these observations.
37

38 284 Mediation analysis further confirmed that *IGF2* methylation as a mediator can
39
40
41 285 account for approximately 21% of the total effect of PFOA exposure on ponderal index at
42
43
44 286 birth, which explains 1.2% of variance in ponderal index. It is remarkable that around
45
46
47 287 one-fifth of the effects of prenatal PFOA exposure on reduced ponderal index can be
48
49
50 288 explained by methylation at only one gene, *IGF2*. However, we only evaluated DNA
51
52
53 289 methylation at three regions, and it is possible that methylation of other unmeasured genes
54
55
56 290 are also responsible for the mediatory effect that we observed. Future epigenome-wide study
57
58
59
60

1
2
3
4 291 would reveal other genes that mediate the effects of prenatal PFOA exposure. Simultaneously,
5
6
7 292 ponderal index is an indicator of fatness and measures the relative soft tissue mass to bone
8
9
10 293 structure. Reduced fetal growth may lead to future health problems such as obesity,
11
12
13 294 hypertension, type II diabetes, and associated disorders. Given the long-term effect of DNA
14
15
16 295 methylation at birth on long-term phenotypic alteration, a study following the participants in
17
18
19 296 our birth cohort who were exposed to PFOA *in utero* is warranted.

20
21 297 In our mediation analysis, we found that *IGF2* methylation had a significant
22
23
24 298 mediatory effect on the relationship between PFOA and ponderal index, despite the negative
25
26
27 299 association between PFOA and ponderal index did not meet statistical significance ($\beta = -0.44$,
28
29
30 300 95% CI: -0.99 to 0.12, $p = 0.123$). In a previous cross-sectional study, Apelberg and
31
32
33 301 colleagues reported a negative association between cord serum PFOA concentration and
34
35
36 302 ponderal index at birth among 293 newborns (39). The cord serum median concentration of
37
38
39 303 PFOA in that study was 1.6 ng/mL, which was similar to or slightly higher than the exposure
40
41
42 304 levels in our study. In addition, the sample size in that study was more than 1.5 times larger
43
44
45 305 than that of our study. Therefore, it is possible that we did not find a significant association
46
47
48 306 between PFOA and ponderal index owing to the limited sample size of our study.
49
50
51 307 Additionally, in cases of mediation analyses, it is possible for an exposure to exert an effect
52
53
54 308 on an outcome indirectly through a mediator, even if we do not observe a significant
55
56
57 309 exposure-outcome association. This can be because of a presence of multiple indirect
58
59
60

1
2
3
4 310 pathways or subpopulations that mask true association (33). Further study with a larger
5
6
7 311 sample size will clarify the association between PFOA exposure and lower ponderal index at
8
9
10 312 birth.

11
12 313 We did not find a significant association between prenatal PFAAs exposure and
13
14
15 314 *LINE1* global methylation. This is consistent with two *in vitro* studies on murine and human
16
17
18 315 cells that showed no significant effects of PFOA exposure on global methylation determined
19
20
21 316
22
23
24 317 measured by flow cytometric immunodetection and LC-MS/MS (40, 41). As mentioned
25
26
27 318 earlier however, Guerrero-Preston and colleagues did show a negative correlation between
28
29
30 319 cord serum PFOA concentrations and cord serum global methylation as determined by
31
32
33 320 ELISA with marginal statistical significance ($p = 0.06$) (22). The mean concentrations of
34
35
36 321 PFOS and PFOA in cord serum in that previous study were 5.8 ng/mL and 1.8 ng/mL
37
38
39 322 respectively, which is similar to the exposure levels observed in our study. However, the
40
41
42 323 different methods for determining global methylation on different target tissues may account
43
44
45 324 for the different results within the two studies. Guerrero-Preston and colleagues (22) used
46
47
48 325 ELISA to determine total methylated cytosine whereas our study measured *LINE1* as a
49
50
51 326 surrogate marker for global methylation. In addition, they analyzed cord serum DNA
52
53
54 327 methylation, which is associated with cell-free DNA circulating in the blood, whereas we
55
56
57 328 analyzed cord blood methylation, which includes leucocyte DNA. Finally, their study

1
2
3
4 329 population contained only 15 pairs of newborns from smoking and non-smoking mothers,
5
6
7 330 and owing to this small sample size, the authors could adjust for only a single variable, either
8
9
10 331 maternal age or gestational age, and may not have adequately controlled for other potential
11
12 332 confounding factors. Future epidemiological studies with larger sample sizes will clarify the
13
14
15 333 effects of PFOA and other PFAAs *in utero* on offspring global DNA methylation.
16
17

18 334 Various factors, including oxidative stress and glutathione depletion, can influence
19
20
21 335 DNA methylation (42). *In vitro* studies have shown that oxidative stress can be increased, and
22
23
24 336 the levels of glutathione depleted, by exposure to PFAAs (43, 44). On the other hand, the
25
26
27 337 epigenetic machinery itself, through DNA methyltransferase activity or histone modification,
28
29
30 338 can cause changes in DNA methylation. PFAAs can act as ligands to activate peroxisome
31
32
33 339 proliferator-activated receptors (PPARs) (45), whose targets include genes encoding
34
35
36 340 histone-modifying enzymes (46). Although the exact mechanism-of-action has yet to be fully
37
38
39 341 elucidated, it is possible that PFAAs modify DNA methylation through binding to PPAR α and
40
41
42 342 γ , and by influencing the epigenetic machinery directly. As is the case for PFAAs, phthalate
43
44
45 343 metabolites such as mono-(2-ethylhexyl) phthalate (MEHP) can act as ligands for PPARs
46
47
48 344 (47). LaRocca and colleagues reported a negative correlation between maternal urinary
49
50
51 345 phthalate metabolites in early pregnancy and placental *IGF2* methylation from 179 placental
52
53
54 346 samples taken from mothers enrolled in two birth cohort studies (48). That finding is in line
55
56
57 347 with our observations on PFOA exposure and reduced *IGF2* methylation in cord blood,
58
59
60

1
2
3
4 348 which may be a consequence of PFOA and MEHP sharing a similar mode of action through
5
6
7 349 PPARs.
8

9
10 350 A stronger association between PFOA exposure and *IGF2* methylation was
11
12 351 observed than for PFOS. Previous studies have shown that PFOA has higher placental
13
14
15 352 transfer efficiency than PFOS does (49, 50). PFOA is also a stronger agonist of PPARs than is
16
17
18 353 PFOS (51). Both of these factors may explain the stronger association of PFOA with *IGF2*
19
20
21 354 methylation.
22
23

24 355 We also found stronger associations of prenatal PFOS exposure with ponderal
25
26
27 356 index than for PFOA exposure yet found only a weak association between PFOS and reduced
28
29
30 357 *IGF2* methylation. Conversely, we observed stronger associations between PFOA and
31
32
33 358 reduced *IGF2* methylation, which mediated some of the association with lower ponderal
34
35
36 359 index. We have previously reported that prenatal PFOS exposure resulted in reduced birth
37
38
39 360 weight among female infants (9). Our recent studies have also indicated that prenatal PFOS
40
41
42 361 exposure results in greater physiological changes than PFOA exposure such as reduced fatty
43
44
45 362 acids levels in pregnant women (10), and thyroid (11) or reproductive hormone imbalance
46
47
48 363 (12) in infants and mothers. Due to these physiological changes during pregnancy, prenatal
49
50
51 364 PFOS exposure may exert a greater effect on birth size when compared with PFOA.
52

53 365 On the other hand, it is possible that PFOA exposure may exert more long-term
54
55
56 366 effects rather than effects observed at birth. We recently reported that prenatal exposure to
57
58
59
60

1
2
3
4 367 PFOA, but not PFOS, had negative associations with the mental developmental index scores
5
6
7 368 of the Bayley Scales of Infant Development second edition (BSID II) among 6-month-old
8
9
10 369 female infants (32). In a Danish birth cohort study, Halldorsson and colleagues reported that
11
12 370 PFOA exposure *in utero* resulted in obesogenic phenotypes and excessive weight gain among
13
14
15 371 20-year-old females (52). These results suggest a long-term effect of PFOA, rather than an
16
17
18 372 effect at birth, and the possibility that DNA methylation might mediate such long-term
19
20
21 373 outcomes. However, we may not have been able to detect subtle effects of PFOS on *IGF2*
22
23
24 374 methylation owing to our small sample size; further studies should be conducted to evaluate
25
26
27 375 our findings.

28
29
30 376 The strength of our study is that it is a birth cohort study that has followed
31
32
33 377 participants from the prenatal period assessing PFAA exposure in maternal blood during
34
35
36 378 pregnancy and DNA methylation in cord blood at birth, which allows interpretations of
37
38
39 379 causal relationships with chronological associations between exposures and outcomes. In
40
41
42 380 addition, measurements of DNA methylation were done by pyrosequencing, which is a
43
44
45 381 high-resolution method to determine site-specific methylation.

46
47 382 We also acknowledge that there are some limitations in our study. First, our birth
48
49
50 383 cohort study is based on a single obstetrics and gynecology hospital. However, the hospital
51
52
53 384 treated the largest number of deliveries in Sapporo city at the time, and when compared to
54
55
56 385 another birth cohort study that covered 37 hospitals in the Hokkaido prefecture, our cohort
57
58
59
60

1
2
3
4 386 did not show any notable differences in the characteristics of mothers and infants (24). Of the
5
6
7 387 pregnant women we initially approached (N=1796), 29% agreed to participate in the birth
8
9
10 388 cohort (N=514). It is possible that some form of self-selection bias may have been introduced
11
12
13 389 at this point. However, as Nilsen and colleagues have suggested, based on comparison of the
14
15
16 390 data from the Norwegian Mother Child Cohort Study (MoBa) with that of the national
17
18
19 391 medical birth registry, a self-selection bias in exposure-outcome association is likely to be
20
21
22 392 small in prospective study designs (53). On the other hand, among our original birth cohort
23
24
25 393 (N=514), the current study population (N=177) was selected for those who have both
26
27
28 394 maternal and cord blood samples, thus limiting the study to mothers who delivered vaginally.
29
30
31 395 Compared to those not included in the study, our study population consisted of younger, as
32
33
34 396 well as a greater number of primiparous, mothers, and infants with longer gestational age,
35
36
37 397 larger birth weight, and length (Table S4), suggesting that the current study population
38
39
40 398 consisted of healthier participants, and may not be a representative of the source population.
41
42
43 399 However, the aim of our study was to evaluate exposure-outcome association. In this context,
44
45
46 400 the associations we observed could be applicable to other populations with a similar exposure
47
48
49 401 range. In fact, it is possible that we underestimated the effect of PFAAs on birth size because
50
51
52 402 the study population comprises healthier participants than those who excluded and had
53
54
55 403 smaller birth size that could potentially be a result of prenatal PFAA exposure (7).

56 404 Secondly, the maternal blood sampling period for PFAA measurements differed
57
58
59
60

1
2
3
4 405 between subjects. Due to an increment of blood volume during pregnancy, PFAAs
5
6 406 concentrations can appear ‘diluted’ as pregnancy progresses even if inherent exposure levels
7
8
9 407 are the same, thus reduce the comparability of PFAAs exposure levels between individuals.
10
11
12 408 However, PFAAs have relatively long half-lives, and we adjusted for the blood sampling
13
14
15 409 period in the multivariate models to minimize this effect on our results. Additionally, we
16
17
18 410 excluded 58 subjects whose maternal blood was collected after birth. These samples were not
19
20
21 411 suitable to assess PFAA exposure levels during pregnancy due to large amount of blood loss
22
23
24 412 at delivery. Although this exclusion further limited sample size of our study, it enabled us to
25
26
27 413 evaluate the effects of prenatal exposure to PFAAs more clearly by excluding potentially
28
29
30 414 unsuitable samples. Thirdly, our study did not have a large enough sample size to maintain
31
32
33 415 statistical power; it is possible therefore that some milder effects of PFAAs on DNA
34
35
36 416 methylation or infant birth size may not have been detected.

37
38
39 417 In conclusion, we found that prenatal PFOA exposure resulted in reduced *IGF2*
40
41
42 418 methylation in cord blood, which in turn was associated with reduced ponderal index at birth.
43
44
45 419 Our mediation analysis suggested that the effects of PFOA on reduced ponderal index were
46
47
48 420 mediated by *IGF2* methylation by approximately 21% of the total effect. Our group
49
50
51 421 previously reported that plasma PFOS and PFOA concentrations had been decreasing in
52
53
54 422 Hokkaido, Japan throughout the 2003-2011 period, whereas concentrations of
55
56
57 423 perfluorononanoic acid (PFNA) and perfluorodecanoic acid (PFDA), have been increasing
58
59
60

1
2
3
4 424 (54). *In vitro* studies have suggested that PFAAs with longer carbon chains, such as PFNA
5
6
7 425 and PFDA, are more potent in activating PPARs and exhibit more cytotoxicity (55, 56).
8
9
10 426 Therefore, it will also be necessary to evaluate the effects of these emerging PFAAs on the
11
12 427 fetal epigenome. As Halldorsson and colleagues have indicated, the effect of prenatal PFOA
13
14
15 428 exposure may permanently modify health aspects of offspring including obesity and being
16
17
18 429 overweight even twenty years later (52). Our result suggests the involvement of DNA
19
20
21 430 methylation in affecting offspring growth at birth upon prenatal PFOA exposure. Following
22
23
24 431 the participants of our birth cohort who were exposed to PFAAs including PFOA, and
25
26
27 432 exhibited lower *IGF2* methylation will elucidate the long-term health effects associated with
28
29
30 433 prenatal PFAAs exposure.
31
32
33 434
34
35

36 435 **Conflict of Interest Statement**

37
38
39 436 The authors declare they have no actual or potential competing financial interests.
40
41
42
43 437
44
45
46 438
47
48
49

50 439 **Acknowledgments**

51
52
53 440 We thank the staff at Sapporo Toho Hospital and the participating mothers and infants for
54
55
56 441 their collaboration. We also thank Y. M. Ito for advice on statistical analyses, T. A. Yila for
57
58
59
60

1
2
3
4 442 advice on epigenetics, and R. Ito and Y. Iwasaki for PFAAs measurements. This study was
5
6
7 443 supported by a Grant-in-Aid for Health Science Research from the Japanese Ministry of
8
9
10 444 Health, Labour and Welfare (No. 717L00204); and by a Grant-in-Aid for Scientific Research
11
12 445 from the Japanese Ministry of Education, Culture, Sports, Science and Technology (No.
13
14
15 446 23390157, 25253050). We also would like to thank Editage (www.editage.jp) for English
16
17
18 447 language editing.
19
20
21 448
22
23
24 449 Supplementary information is available at Journal of Exposure Science and Environmental
25
26
27 450 Epidemiology's website.
28
29
30 451
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

References

1. United Nations Environment Programme. POPRC3: Development of Risk Management Evaluation. 2007 [cited 2015 Jan]. Available from: <http://chm.pops.int/Portals/0/Repository/poprc3/UNEP-POPS-POPRC.3-POPRC-3-5.English.PDF>.
2. Olsen GW, Lange CC, Ellefson ME, Mair DC, Church TR, Goldberg CL et al. Temporal trends of perfluoroalkyl concentrations in American Red Cross adult blood donors, 2000-2010. *Environ Sci Tech* 2012 5; 46: 6330–6338.
3. Lau C, Anitole K, Hodes C, Lai D, Pfahles-Hutchens A, Seed J. Perfluoroalkyl acids: a review of monitoring and toxicological findings. *Toxicol Sci* 2007; 99: 366–394.
4. Olsen GW, Burris JM, Ehresman DJ, Froehlich JW, Seacat AM, Butenhoff JL et al. Half-life of serum elimination of perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. *Environ Health Perspect* 2007; 115: 1298–1305.
5. Inoue K, Okada F, Ito R, Kato S, Sasaki S, Nakajima S et al. Perfluorooctane sulfonate (PFOS) and related perfluorinated compounds in human maternal and cord blood samples: assessment of PFOS exposure in a susceptible population during pregnancy. *Environ Health Perspect* 2004; 112: 1204–1207.
6. Midasch O, Drexler H, Hart N, Beckmann MW, Angerer J. Transplacental exposure of neonates to perfluorooctanesulfonate and perfluorooctanoate: a pilot study. *Int Arch Occup Environ Health* 2007; 80: 643–648.
7. Olsen GW, Butenhoff JL, Zobel LR. Perfluoroalkyl chemicals and human fetal development: an epidemiologic review with clinical and toxicological perspectives. *Repr Toxicol* 2009; 27: 212–230.
8. Verner MA, Luccisano AE, Morken NH, Yoon M, Wu H, McDougall R et al. Associations of perfluoroalkyl substances (PFAS) with lower birth weight: an evaluation of potential confounding by glomerular filtration rate using a physiologically based pharmacokinetic model (PBPK). *Environ Health Perspect* 2015; 123: 1317–1324.
9. Washino N, Saijo Y, Sasaki S, Kato S, Ban S, Konishi K et al. Correlations between prenatal exposure to perfluorinated chemicals and reduced fetal growth. *Environ Health Perspect* 2009; 117: 660–667.
10. Kishi R, Nakajima T, Goudarzi H, Kobayashi S, Sasaki S, Okada E, et al. The association of prenatal exposure to perfluorinated chemicals with maternal essential and long-chain polyunsaturated fatty acids during pregnancy and the birth weight of their offspring: The Hokkaido study. *Environ Health Perspect* 2015; 123: 1038–1045.
11. Kato S, Itoh S, Yuasa M, Baba T, Miyashita C, Sasaki S, et al. Association of perfluorinated chemical exposure in utero with maternal and infant thyroid hormone levels in the Sapporo cohort of Hokkaido study on the environment and children's health. *Environ Health Prev Med* 2016 ahead of pub.
12. Itoh S, Araki A, Mitsui T, Miyashita C, Goudarzi H, Sasaki S, et al. Association of perfluoroalkyl substances exposure in utero with reproductive hormone levels in cord blood in the Hokkaido Study on Environment and Children's Health. *Environ Int* 2016; 94: 51–59.
13. Hackett JA, Surani MA. DNA methylation dynamics during the mammalian life cycle. *Phil Trans*

- 489 *Royal Soc London Series B*. 2013 J5;368(1609):20110328.
- 490 14. Drong AW, Lindgren CM, McCarthy MI. The genetic and epigenetic basis of type 2 diabetes and
491 obesity. *Clin Pharmacol Ther* 2012; 92: 707–715.
- 492 15. Green BB, Marsit CJ. Select prenatal environmental exposures and subsequent alterations of
493 gene-specific and repetitive element DNA methylation in fetal tissues. *Curr Environ Health Rep* 2015; 2:
494 126–136.
- 495 16. Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E et al. Analysis of repetitive
496 element DNA methylation by methylight. *Nuc Acids Res* 2005; 33: 6823–6836.
- 497 17. Gabory A, Jammes H, Dandolo L. The H19 locus: role of an imprinted non-coding RNA in growth
498 and development. *BioEssays* 2010; 32: 473–480.
- 499 18. Bouwland-Both MI, van Mil NH, Stolk L, Eilers PH, Verbiest MM, Heijmans BT et al. DNA
500 methylation of IGF2DMR and H19 is associated with fetal and infant growth: the generation R study. *PloS One*.
501 2013; 8(12):e81731.
- 502 19. St-Pierre J, Hivert MF, Perron P, Poirier P, Guay SP, Brisson D et al. IGF2 DNA methylation is a
503 modulator of newborn's fetal growth and development. *Epigenetics* 2012; 7: 1125–1132.
- 504 20. Cianfarani S. Insulin-like growth factor-II: new roles for an old actor. *Frontiers Endocrinol* 2012;
505 3: 118.
- 506 21. Perkins E, Murphy SK, Murtha AP, Schildkraut J, Jirtle RL, Demark-Wahnefried W, et al.
507 Insulin-like growth factor 2/H19 methylation at birth and risk of overweight and obesity in children. *J*
508 *Pediatrics* 2012; 161: 31–39.
- 509 22. Guerrero-Preston R, Goldman LR, Brebi-Mieville P, Ili-Gangas C, LeBron C, Witter FR et al.
510 Global DNA hypomethylation is associated with in utero exposure to cotinine and perfluorinated alkyl
511 compounds. *Epigenetics* 2010; 5: 539–546.
- 512 23. Kishi R, Kobayashi S, Ikeno T, Araki A, Miyashita C, Itoh S et al. Ten years of progress in the
513 Hokkaido birth cohort study on environment and children's health: cohort profile--updated 2013. *Environ Health*
514 *Prev Med* 2013; 18: 429–450.
- 515 24. Kishi R, Sasaki S, Yoshioka E, Yuasa M, Sata F, Saijo Y et al. Cohort profile: the Hokkaido study on
516 environment and children's health in Japan. *Int J Epidemiol* 2011; 40: 611–618.
- 517 25. Nakata A, Saito K, Iwasaki Y, Ito R, Kishi R, Nakazawa H. Determination of perfluorinated
518 compounds in human milk and evaluation of their transition from maternal plasma. *Bunseki Kagaku* 2009; 58:
519 653–659.
- 520 26. Uh HW, Hartgers FC, Yazdanbakhsh M, Houwing-Duistermaat JJ. Evaluation of regression methods
521 when immunological measurements are constrained by detection limits. *BMC Immunol* 2008; 9: 59.
- 522 27. Lubin JH, Colt JS, Camann D, Davis S, Cerhan JR, Severson RK et al. Epidemiologic evaluation of
523 measurement data in the presence of detection limits. *Environ Health Perspect* 2004; 112: 1691–1696.
- 524 28. Murphy SK, Adigun A, Huang Z, Overcash F, Wang F, Jirtle RL et al. Gender-specific methylation
525 differences in relation to prenatal exposure to cigarette smoke. *Gene* 2012; 494: 36–43.
- 526 29. Bollati V, Baccarelli A, Hou L, Bonzini M, Fustinoni S, Cavallo D et al. Changes in DNA

- methylation patterns in subjects exposed to low-dose benzene. *Cancer Res* 2007; 67: 876–880.
30. Miller HC, Hassanein K. Diagnosis of impaired fetal growth in newborn infants. *Pediatrics* 1971; 48: 511–522.
31. Textor J, Hardt J, Knuppel S. DAGitty: a graphical tool for analyzing causal diagrams. *Epidemiology* 2011; 22: 745.
32. Goudarzi H, Nakajima S, Ikeno T, Sasaki S, Kobayashi S, Miyashita C et al. Prenatal exposure to perfluorinated chemicals and neurodevelopment in early infancy: The Hokkaido study. *Science Total Environ* 2016; 541: 1002–1010.
33. Hayes AF. Introduction to mediation, moderation, and conditional process analysis: a regression-based approach. NY: Guilford Press; 2013.
34. Preacher KJ, Hayes AF. Asymptotic and resampling strategies for assessing and comparing indirect effects in multiple mediator models. *Behav Res Methods* 2008; 40: 879–891.
35. Fairchild AJ, Mackinnon DP, Taborga MP, Taylor AB. R^2 effect-size measures for mediation analysis. *Behav Res Methods* 2009; 41: 486–498.
36. Richiardi L, Bellocco R, Zugna D. Mediation analysis in epidemiology: methods, interpretation and bias. *Int J Epidemiol* 2013; 42: 1511–1519.
37. Watkins DJ, Wellenius GA, Butler RA, Bartell SM, Fletcher T, Kelsey KT. Associations between serum perfluoroalkyl acids and LINE-1 DNA methylation. *Environ Int* 2014; 63: 71–76.
38. Leter G, Consales C, Eleuteri P, Uccelli R, Specht IO, Toft G et al. Exposure to perfluoroalkyl substances and sperm DNA global methylation in Arctic and European populations. *Environ Mol Mut* 2014; 55: 591–600.
39. Apelberg BJ, Witter FR, Herbstman JB, Calafat AM, Halden RU, Needham LL et al. Cord serum concentrations of perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) in relation to weight and size at birth. *Environ Health Perspect* 2007; 115: 1670–1676.
40. Bastos Sales L, Kamstra JH, Cuijckx PH, van Rijt LS, Hamers T, Legler J. Effects of endocrine disrupting chemicals on in vitro global DNA methylation and adipocyte differentiation. *Toxicology in vitro* 2013; 27:1634–1643.
41. Tian M, Peng S, Martin FL, Zhang J, Liu L, Wang Z et al. Perfluorooctanoic acid induces gene promoter hypermethylation of glutathione-S-transferase Pi in human liver L02 cells. *Toxicology* 2012; 296: 48–55.
42. Hitchler MJ, Domann FE. An epigenetic perspective on the free radical theory of development. *Free Radical Biol Med* 2007; 43: 1023–1036.
43. Wielsoe M, Long M, Ghisari M, Bonefeld-Jorgensen EC. Perfluoroalkylated substances (PFAS) affect oxidative stress biomarkers in vitro. *Chemosphere* 2015; 129: 239–245.
44. Hu XZ, Hu DC. Effects of perfluorooctanoate and perfluorooctane sulfonate exposure on hepatoma Hep G2 cells. *Arch Toxicol* 2009; 83: 851–861.
45. Takacs ML, Abbott BD. Activation of mouse and human peroxisome proliferator-activated receptors (alpha, beta/delta, gamma) by perfluorooctanoic acid and perfluorooctane sulfonate. *Toxicol Sci* 2007; 95:

108–117.

46. Wakabayashi K, Okamura M, Tsutsumi S, Nishikawa NS, Tanaka T, Sakakibara I et al. The peroxisome proliferator-activated receptor gamma/retinoid X receptor alpha heterodimer targets the histone modification enzyme PR-Set7/Setd8 gene and regulates adipogenesis through a positive feedback loop. *Mol Cell Biol* 2009; 29: 3544–3555.

47. Desvergne B, Feige JN, Casals-Casas C. PPAR-mediated activity of phthalates: A link to the obesity epidemic? *Mol Cellular Endocrinol* 2009; 304: 43–48.

48. LaRocca J, Binder AM, McElrath TF, Michels KB. The impact of first trimester phthalate and phenol exposure on IGF2/H19 genomic imprinting and birth outcomes. *Environ Res* 2014; 133: 396–406.

49. Beesoon S, Webster GM, Shoeib M, Harner T, Benskin JP, Martin JW. Isomer profiles of perfluorochemicals in matched maternal, cord, and house dust samples: manufacturing sources and transplacental transfer. *Environ Health Perspect* 2011; 119: 1659–1664.

50. Lee YJ, Kim MK, Bae J, Yang JH. Concentrations of perfluoroalkyl compounds in maternal and umbilical cord sera and birth outcomes in Korea. *Chemosphere* 2013; 90: 1603–1609.

51. Vanden Heuvel JP, Thompson JT, Frame SR, Gillies PJ. Differential activation of nuclear receptors by perfluorinated fatty acid analogs and natural fatty acids: a comparison of human, mouse, and rat peroxisome proliferator-activated receptor-alpha, -beta, and -gamma, liver X receptor-beta, and retinoid X receptor-alpha. *Toxicol Sci* 2006; 92: 476–489.

52. Halldorsson TI, Rytter D, Haug LS, Bech BH, Danielsen I, Becher G, et al. Prenatal exposure to perfluorooctanoate and risk of overweight at 20 years of age: a prospective cohort study. *Environ Health Perspect* 2012; 120: 668–673.

53. Nilsen RM, Vollset SE, Gjessing HK, Skjaerven R, Melve KK, Schreuder P et al. Self-selection and bias in a large prospective pregnancy cohort in Norway. *Paediatr Perinat Epidemiol* 2009; 23: 597–608.

54. Okada E, Kashino I, Matsuura H, Sasaki S, Miyashita C, Yamamoto J et al. Temporal trends of perfluoroalkyl acids in plasma samples of pregnant women in Hokkaido, Japan, 2003–2011. *Environ Int* 2013; 60: 89–96.

55. Buhrke T, Kibellus A, Lampen A. In vitro toxicological characterization of perfluorinated carboxylic acids with different carbon chain lengths. *Toxicol Lett* 2013; 218: 97–104.

56. Wolf CJ, Takacs ML, Schmid JE, Lau C, Abbott BD. Activation of mouse and human peroxisome proliferator-activated receptor alpha by perfluoroalkyl acids of different functional groups and chain lengths. *Toxicol Sci* 2008; 106: 162–171.

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 ($P_M = 0.21$).

1
2
3
4 616 Adjusted for maternal age, pre-pregnancy BMI, parity, maternal education, maternal smoking
5
6
7 617 during pregnancy, gestational age, infant sex, maternal blood sampling period.
8
9
10 618 *ab*: indirect effect, BCa CI: bias-corrected and accelerated confidence interval, P_M: percent
11
12
13 619 mediation
14
15 620 * $p < 0.05$, ** $p < 0.01$
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

	Detection	Detection	Mean	Geometric	Percentile				
	limit	Rate (%)†	(±SD)	Mean	Min	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).

	Mean ±SD	PFOS (ng/ml)		PFOA (ng/ml)	
		ρ	p	ρ	p
		Median (25th-75th)		Median (25th-75th)	
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**

633

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

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

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

637 *p < 0.05, **p < 0.01

638

639

640

Table 3. Association between maternal PFAA concentrations and cord blood DNA

methylation in multiple linear regression analyses (N=177).

	<i>IGF2</i> methylation (%)		<i>H19</i> methylation (%)		<i>LINE1</i> 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
	<i>R</i> ² = 0.056		<i>R</i> ² = 0.018		<i>R</i> 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
	<i>R</i> ² = 0.072		<i>R</i> ² = 0.018		<i>R</i> 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 ($P_M = 0.21$).

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

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

Supplementary Information

Contents

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

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

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

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

Fig S1. Flow chart of study participant selection.

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	chr1 lp15.5, 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 ‡	chr1 lp15.5, site 1: 1,964,261; site 2: 1,964,259; site 3: 1,964,257; site 4: 1,964,254	38 µl 10 µl	30.4 ng 8.3 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
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).

	N	<i>IGF2</i> (%)		<i>H19</i> (%)		<i>LINE1</i> (%)	
		ρ		ρ		ρ	
		Median (25th-75th)	p	Median (25th-75th)	p	Median (25th-75th)	p
DNA methylation (Mean $\pm$SD)	177	48.5 $\pm$ 3.0		52.3 $\pm$ 2.0		75.8 $\pm$ 1.1	
Maternal characteristics							
Maternal age (year) ^a	177	$\rho = 0.148$	0.050*	$\rho = 0.146$	0.053	$\rho = 0.146$	0.053
Pre-pregnancy BMI (kg/m ²) ^a	176 [†]	$\rho = 0.085$	0.26	$\rho = -0.081$	0.287	$\rho = -0.059$	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	$\rho = 0.057$	0.451	$\rho = 0.02$	0.794	$\rho = 0.066$	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	$\rho = 0.112$	0.139	$\rho = 0.043$	0.574	$\rho = 0.055$	0.472
Birth length (cm) ^a	176 [†]	$\rho = -0.079$	0.300	$\rho = 0.066$	0.386	$\rho = 0.138$	0.068
Ponderal Index (kg/m ³) ^a	176 [‡]	$\rho = 0.222$	0.003**	$\rho = -0.036$	0.640	$\rho = -0.110$	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	<i>Ref.</i>		53	≤ 0.9	<i>Ref.</i>	
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
<i>p for trend</i>			<i>p = 0.437</i>				<i>p = 0.007**</i>	

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.