Association of prenatal acetaminophen use and acetaminophen metabolites with DNA methylation of newborns: analysis of two consecutive generations of the Isle of Wight birth cohort

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Abstract

Acetaminophen is used by nearly two-thirds of pregnant women. Although considered safe, studies have demonstrated associations between prenatal acetaminophen use and adverse health outcomes in offspring. Since DNA methylation (DNAm) at birth may act as an early indicator of later health, assessments on whether DNAm of newborns is associated with gestational acetaminophen use or its metabolites are needed. Using data from three consecutive generations of the Isle of Wight cohort (F0-grandmothers, F1-mothers, and F2-offspring) we investigated associations between acetaminophen metabolites in F0 serum at delivery with epigenome-wide DNAm in F1 (Guthrie cards) and between acetaminophen use of F1 and F2-cord-serum levels with F2 cord blood DNAm. In epigenome-wide screening, we eliminated non-informative DNAm sites followed by linear regression of informative sites. Based on repeated pregnancies, indication bias analyses tested whether acetaminophen indicated maternal diseases or has a risk in its own right. Considering that individuals with similar intake process acetaminophen differently, metabolites were clustered to distinguish metabolic exposures. Finally, metabolite clusters from F1-maternal and F2-cord sera were tested for their associations with newborn DNAm (F1 and F2). Twenty-one differential DNAm sites in cord blood were associated with reported maternal acetaminophen intake in the F2 generation. For 11 of these cytosine-phosphate-guanine (CpG) sites, an indication bias was excluded and five were replicated in F2 with metabolite clusters. In addition, metabolite clusters showed associations with 25 CpGs in the F0-F1 discovery analysis, of which five CpGs were replicated in the F2-generation. Our results suggest that prenatal acetaminophen use, measured as metabolites, may influence DNAm in newborns.

Key words: acetaminophen metabolites; cohort studies; Isle of Wight; pharmacoepidemiology; epigenetic; DNA methylation

Introduction

Acetaminophen (N-acetyl-4-aminophenol), also known as paracetamol, is considered a safe analgesic and antipyretic medication for pregnant women [1]. Approximately two-thirds of women use acetaminophen during pregnancy, half of them in the first trimester [2–5]. Although acetaminophen is classified as a category B drug by the US Food and Drug Administration (i.e. no risks observed in pregnant women), no randomized controlled trials have been reported. Moreover, experimental animal models of acetaminophen effects are inconsistent [6].

Prenatal acetaminophen exposure was demonstrated to be associated with adverse neurological and cognitive learning as well as impaired lung in children [7–9]. Acetaminophen metabolism is altered during the pregnancy which might make women and their fetuses susceptible to toxicity due to increased fraction of oxidative metabolite N-acetyl-p-benzoquinone imine [10, 11]. Acetaminophen and its metabolites can enter the placenta, they are found in cord blood, newborn’s urine, and the fetal liver [1, 12]. It is mostly metabolized in the liver by conjugation with glucuronic (45–55%) and sulfuric acid (30–35%) [13]. Less than 5–10% is metabolized by hepatic cytochrome P450 enzymes to the highly reactive intermediate N-acetyl-p-benzoquinonimine (NAPQI), which is quickly detoxified to an N-acetylcysteine conjugate; less than 5% of acetaminophen is excreted unchanged in urine [13].

Prior studies have suggested that DNA methylation (DNAm) in placental, cord blood, and blood samples may act as a mediator between gestational acetaminophen exposure and asthma and neurodevelopmental disorders in childhood [14–22]. Methylation of cytosine-guanine dinucleotides (CpGs) can be identified in...
and 234 maternal serum samples were collected at delivery for DNAm assessment. For the F2-generation, 194 cord blood DNA samples were separated and stored at −80°C. In total, 796 Guthrie cards were analyzed for acetaminophen metabolites. In the F1-generation, information on acetaminophen use and on analyses of acetaminophen metabolites in sera of their F0-mothers collected at birth and DNAm from Guthrie cards in F1. In F2-newborns, available data included F1-maternal information on acetaminophen use during pregnancy, past medical history, metabolites in F2-cord sera, and DNAm measured in F2-cord blood.

Methods

IOW Birth Cohorts

Study participants were recruited between 1989 and 1990 by contacting potential parents (first generation, IOW-F0). Approximately, 95% of infants (n = 1456) were enrolled after exclusion of perinatal deaths, adoptions, moving out, and refusals. The main goals of the study were to identify genetic, environmental, and epigenetic risk factors for asthma and allergies. The second generation (F1) has been followed up for 26 years. The third generation (F2-children of the F1 birth cohort, n = 543) has been recruited since 2010 during pregnancies of F1-females or partners of F1-males. When F1-women or partners became pregnant, information was gathered about their lifestyle and health during pregnancy. The IOW Birth Cohort and Third Generation Cohort were approved by the local research ethics committee (NRES Committee South Central—Hampshire B, U.K.) and the University of Memphis Institutional Review Board (STUDY #: 2423). Written consents were obtained from all participants or their parents at recruitment and all follow-ups.

Acetaminophen during Pregnancy

For the F0-generation, no information on acetaminophen use was collected during pregnancy. However, serum samples of F0 collected at the end of the pregnancy were analyzed for acetaminophen metabolites. In the F1-generation, information on the use of medication was collected twice during pregnancy using questionnaires before and after week 20. In addition, cord sera of F2 were analyzed for acetaminophen metabolites.

Collection of Blood Samples in F1 and in F2

Blood samples were obtained from 1056 F0-mothers at delivery; sera were separated and stored at −80°C. Within 7 days after birth, heel blood samples from F1-newborns were collected onto filter paper (Guthrie cards). In total, 796 Guthrie cards were analyzed for DNAm assessment. For the F2-generation, 194 cord blood DNA and 234 maternal serum samples were collected at delivery.

Acetaminophen Metabolites

Metabolites, nutrients, and toxins (MNTs) were analyzed using a deep data-independent untargeted approach based on high-resolution mass spectrometry coupled to varied sample introduction strategies (reversed-phase liquid chromatography, hydrophilic interaction liquid chromatography, and flow-injection analysis). These strategies enable blind molecular profiling that does not require a priori knowledge of compound identities and is suitable for detecting a wide range of substances derived from environment, food, gut microbiome, and endogenous exposures.

Serum samples (MNTs) were analyzed in random order, with each batch including analyses of multiple blanks, pooled quality control extracts, and extracts of a reference serum. Profiling of polar fractions of serum metabolites was performed using a Thermo QExactive mass spectrometer interfaced to a Thermo Vanquish Binary Flex pump and autosampler. Metabolite analyses were conducted using positive-ion mode electrospray ionization. Procedural details are described in Supplemental Materials. Mass spectra were acquired using all ion fragmentation. Peak areas exported from Progenesis QI software (Waters Corp., Milford, MA USA) software were filtered to remove signals with the highest abundances in blanks and those with relative mass defect (RMD) > 1200 ppm [27, 28], as these are often attributable to inorganic salts. Acetaminophen metabolites were annotated based on exact mass matches (Δm < 3 ppm), isotopolog abundance matching to theoretical values, observation of characteristic fragment ions, and coelution with commercially available authentic standards. Variability in levels (CV) measured in a reference serum across all batches was signal-dependent but < 20% for acetaminophen sulfate after exclusion of a single outlier batch. Metabolite concentrations were calculated from peak areas relative to a labeled internal standard (cotinine-d5) and converted to μg/L using empirical relative response factors for available authentic standards.

DNA Methylation Measurement

In F1, DNA was extracted from dried blood spots on Guthrie cards using QIAamp DNA Investigator kits (Qiagen, Germantown, MD, USA) following the manufacturer’s instructions. Concentrations of DNA were determined by Qubit spectrophotometry. For details see the Supplemental Materials. Measurements of genome-wide DNAm were performed using the Illumina MethylationEPIC 850K platform (Illumina, Inc., CA, USA) which interrogates >850,000 CpGs associated with over 24,000 genes. Data were preprocessed and corrected for batch and cell-type proportion according to published procedures to prepare DNA methylation for statistical analysis (see Supplementary Material for details) [29–34].

Statistical Analyses and Their Flow

The statistical analyses in Fig. 1 were performed using SAS/STAT® software, version 9.4 of the SAS system (SAS Institute, Cary, NC, USA) and version 64 3.5.2R (The R Foundation for Statistical Computing, Vienna, Austria). First, we conducted epigenome-wide association assessments to identify whether newborns’ CpGs were associated with F1 maternal acetaminophen questionnaire information (Fig. 1) using the ttScreening R package (v1.5, http://cran.r-project.org/web/packages/ttScreening/) [26]. This method removes non-informative CpGs in a course of 100 repetitions of a training-and-testing process with robust regressions. CpGs were considered informative if they showed statistical significance in both training and testing samples for 75 out of
100 repetitions. Since reported acetaminophen use was available only in F1-F2 mother–offspring dyads, Fig. 1 shows that we started with information on gestational use in 183 F1-F2 pairs, then analyzed clusters of acetaminophen metabolites in 493 F0-F1 mother–offspring dyads. Following each t-Screening, informative CpGs were tested using generalized linear regression models adjusting for potential confounders. In these steps, we applied Bonferroni corrections for multiple testing of informative CpG in F0-F1 and in F1-F2.

Next, analyses of an indication bias (acetaminophen may indicate an underlying disease) were performed using repeated pregnancies for selected CpG sites that survived prior linear regression. To investigate indication biases [35], two nested pregnancy control analyses were conducted in the F2-generation, first, for maternal reports of acetaminophen use (n = 48 pregnancies in 24 F1-women) and second, for clusters of metabolites (n = 52, four additional observations, 26 F1-women). We investigated whether the associations are truly related to acetaminophen or whether it indicates an underlying disease. Following a model proposed by Shaheen et al. [35], we investigated information of the same F1-mothers with identical or varying medical conditions of diseases and acetaminophen in repeated pregnancies (Fig. 1). In this step, we used linear mixed models in SAS (SAS Institute, Cary, NC, USA). Given the reduced statistical power due to smaller sample sizes, a P-value of 0.10 was used to determine statistically significant acetaminophen effects controlling for chronic diseases.

Given that individuals with similar acetaminophen intake may metabolize it differently, we clustered the six metabolites (PROC FASTCLUS, SAS Institute, Cary, NC, USA). For both, serum levels in F0 and F2 (Fig. 1), these analyses provided clusters with multiple levels of metabolites. Metabolite clusters were then used first, to determine the maximum levels of metabolites in the cluster with lowest metabolite levels (non-exposed cluster), and second, to discover associations of metabolite clusters with DNA methylation in the F1- and F2-generations. The first step provided us information on how to categorize metabolites (exposed or not). This dichotomization was applied to determine sensitivity and specificity of the questionnaire data. Second, in both F0 and F2 serum samples, levels of all six acetaminophen metabolites were correlated (Figure S1). Nevertheless, they may still present differing patterns due to distinct individual metabolism and elapsed times between acetaminophen consumption and serum collection. Hence, we identified combinations of acetaminophen metabolites (clusters), separately for F0 and F2, and used clusters with higher values as exposed groups. In F2, to estimate sensitivity and specificity of

Figure 1: Flow chart of the statistical analyses
Table 1: Comparison of maternal and newborn characteristics and covariates between the initial and the analytical sample within the IOW cohort for F0-F1 mother–offspring dyads

| Variable                      | Original cohort n = 1456 (%) | Analytical sample n = 493 (%) | P value |
|-------------------------------|------------------------------|-------------------------------|---------|
| Sex                           | Boy                          | 50.8                          | 50.2    | 0.92    |
|                               | Girl                         | 49.1                          | 49.7    |         |
| Prenatal smoking              | Yes                          | 19.7                          | 4.7     | 0.009   |
| Maternal asthma               | Yes                          | 8.1                           | 2.7     | 0.79    |
| Season of Birth               | Spring                       | 37.9                          | 31.2    | 0.001   |
|                               | Summer                       | 31.2                          | 34.5    |         |
|                               | Fall                         | 30.7                          | 34.2    |         |
| Socioeconomic status          | Low                          | 39.7                          | 35.9    | 0.65    |
|                               | Medium                       | 34.1                          | 33.9    |         |
|                               | High                         | 25.9                          | 30.2    |         |
| Birth order                   | First born                   | 32.5                          | 20.2    | 0.001   |
|                               | Second born                  | 23.7                          | 28.8    |         |
|                               | Third born or more           | 43.8                          | 51.0    |         |

*a Categorical variables were tested using chi-square goodness of fit test. Continuous variables were tested using one-sample t-test. All variables were compared with 'No' as a reference.

reported use, groups exposed to higher metabolite values were compared to reported acetaminophen.

Confounders

In F0-F1, confounders were gestational smoking, maternal asthma, birth order, gender of the child, and season of birth. Information was collected by interviews and by hospital records (gender and month of birth). In F1-F2, covariates were newborn gender, maternal chest infections during pregnancy, bacterial infection, urinary tract infection assessed from hospital records, maternal smoking during pregnancy, socioeconomic status (SES), newborn birth order, chronic diseases (asthma, n = 6, gestational diabetes, arthritis, and polycystic ovaries each in two pregnancies, anxiety and depression, autoimmune thyroid disease and sacral nerve damage each in one pregnancy), having a pet during pregnancy, and damp spots on houses’ walls (interviews). In addition, in analyses of CpGs in F1 and F2, the proportions of different blood cells were controlled for when assessing effects on differential DNAm.

Results

Participant Demographic and Characteristics

F0-mothers of 1456 F1-offspring completed the questionnaire during pregnancy. Among 493 F1-newborns for whom biochemical markers and DNAm were available (analytical sample), 50.2% were boys and 49.7% were girls (Table 1). A total of 543 female F1-participants or female partners of the male participants completed the questionnaire while pregnant with F2 (Table 2). DNAm and acetaminophen metabolite data were available for 183 F2-newborns. In this analytical sample, acetaminophen use was reported in 64.4% of early (first trimester) and in 57.8% during late pregnancy (second and third trimesters); in 38.8% acetaminophen use occurred in all trimesters. In contrast, acetaminophen sulfate, often the most abundant metabolite, was detected in 76% of F2 sera. In F0, it was detected in 91% of the samples. Median serum levels of this metabolite were slightly lower in F0 relative to F2, perhaps suggesting a small amount of decomposition during about 30 years of serum storage (Figure S2). Table 3 shows the timing of gestational acetaminophen use and maternal and newborn characteristics. Compared to non-users, the median concentrations of five acetaminophen metabolites in cord serum were higher in acetaminophen users except for cysteine conjugate, although the differences were not statistically significant (Table 4).

Differentially Methylated CpGs Associated with Reported Use and Acetaminophen Metabolites

In F1-F2 mother–offspring dyads, using epigenome-screening and subsequent linear regression, 21 informative CpGs were discovered to be associated with reported acetaminophen use (Table S1). To investigate indication biases, we analyzed repeated pregnancies in the same women. This sample includes 24 women with at least two pregnancies (n = 48, Table S2). A repeated pregnancy analysis with simultaneous information on acetaminophen and chronic diseases showed that 11 of the 21 discovered CpGs remained statistically significantly associated with reported acetaminophen (Table 5). Given that this sub-analysis is based on a small sample, a significance level of 0.10 was used. In these analyses, estimates of acetaminophen showed the same direction as in the prior linear regressions.

In a ‘look-up’ analysis, we tested the 11 CpGs identified to be related to acetaminophen use and survived the indication bias assessment (Table 5) for associations with the metabolite clusters in F1-F2 dyads. Five CpGs were found to be statistically significant related to metabolite clusters (Table S6) including the SCFD2, the LINC00589, the CCDC109B, the ARAP1, and the CASPS5 genes.

In F2, the maximum metabolite values of the cluster with the lowest concentration of metabolites were used as cut-off points (Table S3, cluster II). Based on these cut-offs, the sensitivity of reported acetaminophen use during gestation was 71.31% and the specificity was 38.1% (Table S4). Interestingly, clustering of the metabolites found in F0-sera produced similar cluster profiles (Table S5, ‘high levels of all metabolites’ and ‘high levels except cysteine conjugate’).

Next, using metabolite clusters in F0-serum we investigated whether two metabolite clusters and F1-newborns CpGs were associated using epigenome-wide screening. With a selection probability ≥ 75%, 51 CpGs were discovered (Table S7). These 51 CpGs were then tested for associations with two exposure clusters (one with higher levels of all metabolites and one with higher levels except cysteine conjugate, Table S5, clusters V and IV). Using generalized linear models and Bonferroni adjustment (Table 6), 25 of 51 CpGs were statistically significantly linked to acetaminophen metabolite clusters. The 25 CpGs were examined for replication in F2 using metabolite clusters (Table S3) employing P-values < 0.1.
Table 2: Comparison of maternal and newborn characteristics and covariates between the initial and the analytical sample within the IOW cohort for F1-F2 mother-offspring dyads

| Variable                              | Original cohort n = 543 (%) | Analytical sample N = 183 (%) | P value |
|---------------------------------------|----------------------------|-------------------------------|---------|
| Acetaminophen uses during Pregnancy\(b\) | Early pregnancy 51.2        | 64.4                           | 0.74    |
|                                       | Late pregnancy 39.4         | 57.8                           |         |
|                                       | Early or late pregnancy 31.4| 29.5                           |         |
|                                       | Both, early and late pregnancy 29.5 | 38.8 |         |
| Sex                                   | Boy 55.6                    | 51.4                           | 0.99    |
|                                       | Girl 44.4                   | 48.6                           |         |
| Prenatal smoking                      | 1–10 14.7                   | 16.9                           | 0.72    |
|                                       | >10 19.7                    | 21.3                           |         |
| Chronically ill during pregnancy      | Yes 25.9                    | 35.0                           | 0.02    |
| Birth order                           | First born 51.8             | 49.2                           | 0.67    |
|                                       | Second born 29.9            | 33.3                           |         |
|                                       | Third born or more 18.3     | 17.50                          |         |
| Infectious disease during pregnancy   | Yes 10.0                    | 12.6                           | 0.35    |
| Socioeconomic status                  | Low 19.4                    | 14.2                           | 0.16    |
|                                       | Medium 68.0                 | 61.2                           |         |
|                                       | High 12.5                   | 24.6                           |         |
| Having pet during pregnancy           | Yes 53.5                    | 54.6                           | 0.79    |
| Season of Birth\(c\)                 | Spring 25.9                 | 23.50                          | 0.75    |
|                                       | Summer 27.6                 | 26.8                           |         |
|                                       | Fall 24.6                   | 24.0                           |         |
| Damp spots in houses’ wall            | Yes 35.5                    | 37.70                          | 0.60    |

\(a\) Categorical variables were tested using chi-square goodness of fit test. Continuous variables were tested using one-sample t-test. All variables were compared with ‘No’ as a reference.

\(b\) Self-reported acetaminophen use during pregnancy within the IOW cohort was characterized based on any maternal acetaminophen use (early or late pregnancy) compared to no use.

\(c\) Winter was used as a reference.

Five CpGs were found to have an association to the cluster with ‘high level of all metabolites except cysteine conjugate’ in F2 (Table 7). Next, the indication bias was assessed using repeated pregnancies controlling for maternal identification. Of the 52 pregnancies, two exposure clusters were present in 7 and 11 pregnancies without a chronic disease (Table S8). Controlling for indication bias, two CpGs remained associated with the respective cluster (Table 8).

**Discussion**

Of 21 CpGs associated with acetaminophen use, 11 CpGs remained associated when a potential indication bias due to chronic diseases was considered. We detected acetaminophen and five metabolites in serum samples: non-metabolized acetaminophen, acetaminophen glucuronide and cysteine conjugate, and N-acetyl cysteine conjugate (annotations presented in Supplementary Material, Table S10). In addition, two metabolites of the sulfation pathway were seen: acetaminophen sulfate and 2-hydroxyacetaminophen sulfate [36]. Since the metabolites were associated, we clustered them and used two clusters as exposures: one cluster with ‘high levels except cysteine conjugate’ and one cluster with ‘high levels of all metabolites’. Regarding F0-serum metabolites and F1-DNAm, we discovered 25 CpGs linked to one of the two metabolite clusters. Of the 25 CpGs, five CpGs were replicated in the F2-generation (RHH, TRAF7, MBNL2, COX4NB, UBE3C genes). Two the 25 CpGs could not be explained by an indication bias in the F2-generation. One, namely cg06155414 (MBNL2), was also among those CpGs detected in F0-F1 and replicated in F2 (Tables 7 and 8).

Five specific genes of 11 CpGs related to reported acetaminophen use in F1-F2 dyads (Table 5), not eliminated when checking an indication bias and also associated with the metabolites clusters (Table S6), suggest that acetaminophen during pregnancy could lead to differential DNAm of genes related to respiratory disorders in other studies (SCFD2) [37], proposing an explanation for associations of wheezing and childhood asthma with acetaminophen [38–41]. LINCO0589 gene encodes for IncRNAs, which can regulate interleukin 6 (IL-6) and signal transducer and activator of transcription 3 (STAT3) [42]. Upregulation of the CCDC109B gene, also called MCUB, may impair mitochondrial energy production via glucose oxidation and is related to type 2 diabetes mellitus [43]. In addition, also the ARAP1 gene (Table 5, Table S6) suggest potential associations with diabetes mellitus [44, 45]. CASP5 belongs to the group of inflammatory caspases [46].

Of the 25 CpGs related to acetaminophen metabolite clusters in F1 (Table 6), five CpGs were replicated in F2 (Table 7). Two CpGs on the ITGB2 and MBNL2 genes survived the indication bias analysis (Table 8). The ITGB2 gene encodes integrin beta chain that works in cell adhesion as well as cell-surface mediated signaling. The gene plays important role in immune response and is associated in leukocyte adhesion deficiency [47]. The MBNL2 gene encodes a protein which mediates pre-mRNA alternative splicing regulation. This gene seems to be the primary splicing factor sequestered by toxic RNAs in the brain of patients with myotonic dystrophy and that loss of this factor are underlies the neurological features associated with myotonic dystrophy [48]. To summarize, potential genetic associations of the identified CpGs show a variety of potential outcomes, which need to be tested in future studies.

The median concentrations of the metabolites in F2-cord serum (Table S9) suggest that 66% (F1: 75%) is metabolized to sulfate, 15% (F1: 11%) to glucuronide, and 12.86% (F1: 0.08%) to acetylcysteine conjugates. This is different from nonpregnant settings with conjugation of glucuronic (45–55%) and sulfuric acid (30–35%) [13, 49], and suggests that in the fetus (or during pregnancy), acetaminophen is metabolized differently, as reported in the literature [50, 51].
### Table 3: F1-maternal and F2-newborn characteristics by self-reported acetaminophen use during pregnancy within the IOW cohort (N = 183)

| Variable                              | Any acetaminophen use<sup>a</sup> | Acetaminophen use in first trimester | Acetaminophen use in last trimester |
|---------------------------------------|-----------------------------------|-------------------------------------|-------------------------------------|
|                                       | Yes (%) (n = 112) | No (%) (n = 58) | P value | Yes (%) (n = 118) | No (%) (n = 65) | P value | Yes (%) (n = 78) | No (%) (n = 57) | P value |
| Offspring sex                         |                                  |                                     |         |                  |                  |         |                  |                  |         |
| Boy                                   | 33.5                             | 17.0                                | 0.46    | 32.3             | 18.3             | 0.71    | 28.3             | 21.7             | 0.71    |
| Girl                                  | 35.4                             | 14.0                                |         | 32.9             | 16.5             |         | 30.0             | 20.0             |         |
| Prenatal smoking                      |                                  |                                     |         |                  |                  |         |                  |                  |         |
| 1–10                                  | 12.8                             | 4.3                                 | 0.64    | 12.2             | 4.9              | 0.73    | 13.3             | 5.9              | 0.32    |
| >10                                   | 15.2                             | 6.1                                 |         | 14.0             | 7.3              |         | 14.2             | 8.3              |         |
| Chronically ill during pregnancy      |                                  |                                     |         |                  |                  |         |                  |                  |         |
| Yes                                   | 25.6                             | 7.9                                 | 0.14    | 23.8             | 9.8              | 0.28    | 19.2             | 15.0             | 0.72    |
| Birth order                           |                                  |                                     |         |                  |                  |         |                  |                  |         |
| First born                            | 30.5                             | 15.2                                | 0.41    | 29.3             | 16.5             | 0.80    | 25.0             | 19.2             | 0.14    |
| Second born                           | 25.6                             | 10.4                                |         | 24.4             | 11.6             |         | 24.2             | 14.2             |         |
| Third born or more                    | 12.8                             | 5.5                                 |         | 11.6             | 6.7              |         | 9.2              | 8.3              |         |
| Infectious disease during pregnancy   |                                  |                                     |         |                  |                  |         |                  |                  |         |
| Yes                                   | 7.3                              | 5.5                                 | 0.21    | 6.7              | 6.1              | 0.19    | 7.5              | 7.5              | 0.44    |
| Socioeconomic status                  |                                  |                                     |         |                  |                  |         |                  |                  |         |
| Low                                   | 8.7                              | 5.5                                 | 0.81    | 8.7              | 5.5              | 0.77    | 3.0              | 6.7              | 0.06    |
| Medium                                | 42.6                             | 18.6                                |         | 39.3             | 21.9             |         | 41.5             | 23.0             |         |
| High                                  | 16.9                             | 7.7                                 |         | 16.4             | 8.2              |         | 13.3             | 12.0             |         |
| Having pet                            |                                  |                                     |         |                  |                  |         |                  |                  |         |
| Yes                                   | 36.6                             | 18.0                                | 0.68    | 33.3             | 21.3             | 0.28    | 37.0             | 26.0             | 0.75    |
| Season of birth                       |                                  |                                     |         |                  |                  |         |                  |                  |         |
| Spring                                | 15.8                             | 7.6                                 | 0.25    | 14.2             | 9.3              | 0.46    | 14.0             | 11.1             | 0.96    |
| Summer                                | 19.7                             | 7.1                                 |         | 19.1             | 7.6              |         | 16.3             | 12.0             |         |
| Fall                                  | 18.0                             | 6.0                                 |         | 16.4             | 7.6              |         | 13.3             | 8.15             |         |
| Damp spots in houses’ wall            |                                  |                                     |         |                  |                  |         |                  |                  |         |
| Yes                                   | 23.5                             | 14.2                                | 0.18    | 22.4             | 15.3             | 0.27    | 23.0             | 19.3             | 0.50    |

<sup>a</sup>Covariates include newborns sex, smoking level during pregnancy, SES, having pet during pregnancy, season of birth, infectious and chronic disease, order of birth, and damp spots on houses’ walls. Categorical variables were tested using chi-square goodness of fit test. Continuous variables were tested using one-sample t-test.

<sup>b</sup>Self-reported acetaminophen use during pregnancy within the IOW cohort was characterized based on any maternal acetaminophen use (early or late pregnancy) compared to no use.

<sup>c</sup>Infections include bacterial, chest, and/or urinary tract infection based on hospital records.

<sup>d</sup>‘Winter’ was used as a reference.

### Table 4: Distribution of acetaminophen use during pregnancy and metabolites (microgram/L) in F2-serum (N = 163)

| Metabolites in F2-serum               | Acetaminophen use throughout pregnancy | Acetaminophen use in the first half of the pregnancy | Acetaminophen use in the last half of the pregnancy |
|---------------------------------------|---------------------------------------|----------------------------------------|---------------------------------|
|                                       | Yes (n = 51) | Median | IQR<sup>a</sup> | No (n = 112) | Median | IQR<sup>a</sup> | Yes (n = 57) | Median | IQR<sup>a</sup> | No (n = 106) | Median | IQR<sup>a</sup> | Yes (n = 50) | Median | IQR<sup>a</sup> |
| Acetaminophen sulfate (1.15_231.0197 n) | 15.54 | 4477.0 | 0.86 | 73.01 | 0.31 | 20.2 | 127.3 | 0.86 | 73.01 | 0.27 | 12.88 | 131.5 | 0.0 | 34.36 | 0.95 |
| 2-hydroxy acetaminophen sulfate (1.07_247.0146 n) | 1.73 | 18.84 | 0.99 | 14.77 | 0.26 | 1.76 | 20.26 | 1.00 | 14.77 | 0.21 | 1.78 | 16.22 | 1.25 | 23.4 | 0.71 |
| Acetaminophen glucuronide (7.41_327.0946 n) | 3.2 | 18.84 | 0.00 | 13.32 | 0.14 | 3.67 | 18.88 | 0.0 | 12.67 | 0.12 | 3.52 | 18.25 | 1.97 | 12.67 | 0.6 |
| Cysteine conjugate [6.97 271.0741 m/z] | 0.0 | 0.38 | 0.0 | 0.11 | 0.09 | 0.0 | 0.37 | 0.0 | 0.11 | 0.08 | 0.0 | 0.4 | 0.0 | 0.25 | 0.48 |
| N-acetyl cysteine conjugate [5.11 313.0846 m/z] | 2.0 | 16.79 | 0.19 | 8.46 | 0.06 | 2.0 | 18.37 | 0.19 | 8.46 | 0.07 | 2.05 | 14.04 | 1.02 | 10.15 | 0.38 |
| Acetaminophen [1.15 152.0705 m/z] | 0.73 | 6.2 | 0.21 | 3.94 | 0.31 | 0.81 | 6.28 | 0.21 | 3.94 | 0.27 | 0.69 | 4.79 | 0.34 | 8.03 | 0.95 |

<sup>a</sup>IQR, interquartile range.
Table 5: Effect of acetaminophen use in repeated pregnancies of the same women with varying underlying diseases and varying use of acetaminophen use in F1 with CpG sites in the F2-generation (n = 48)

| CpG        | UCSC reference gene name | Estimate | P value | Early or late prenatal acetaminophen vs. no acetaminophen | Estimate | P value |
|------------|--------------------------|----------|---------|----------------------------------------------------------|----------|---------|
| cg06312846 | FOXF2, FOXQ1             | −0.01488 | 0.4437  |                                                          | −0.03697 | 0.0315  |
| cg20601329 | SCFD2                    | −0.00866 | 0.0076  |                                                          | −0.00753 | 0.0073  |
| cg23674011 | LINC00589                | −0.00383 | 0.6483  |                                                          | −0.01542 | 0.00379 |
| cg16105461 | DAGLA                    | 0.01220  | 0.2566  |                                                          | 0.02097  | 0.0272  |
| cg17756860 | CCDC109B                 | 0.01983  | 0.0581  |                                                          | 0.02101  | 0.0214  |
| cg27321325 | ARAP1                    | −0.01103 | 0.0301  |                                                          | −0.0782  | 0.0727  |
| cg04944682 | ACTR3BP2                 | 0.01904  | 0.4194  |                                                          | 0.03646  | 0.0931  |
| cg16008631 | RPH3AL                   | −0.00395 | 0.6483  |                                                          | −0.00753 | 0.0073  |
| cg11905407 | CASP5                    | 0.01068  | 0.0602  |                                                          | 0.01100  | 0.0264  |
| cg21909643 | CDKAL1                   | −0.01034 | 0.3043  |                                                          | −0.01667 | 0.0589  |

Table 6: Association of metabolites clusters in maternal F0-serum with differential methylation of CpGs in F1-newborns cord blood using linear regression models adjusting for confounders

| CpG        | UCSC reference gene name | Estimate | P value | Cluster with high metabolite levels except cysteine conjugate (n = 77, reference n = 252) | Estimate | P value |
|------------|--------------------------|----------|---------|------------------------------------------------------------------------------------------|----------|---------|
| cg19269807 | KCNK9                    | 0.005861 | 3.6E-06 |                                                                                         | 0.001687 | 0.2482  |
| cg02268314 |                           | 0.037896 | 5.25E-06|                                                                                         | 0.003251 | 0.7389  |
| cg02667752 | SKA2                     | −0.01559 | 1.25E-05|                                                                                         | −0.00114 | 0.7585  |
| cg02117924 | ITGB2                    | −0.0204  | 1.45E-05|                                                                                         | −0.00119 | 0.815   |
| cg20563193 |                           | −0.02187 | 2.27E-05|                                                                                         | −0.01182 | 0.0389  |
| cg08781655 | MAP3K4                   | −0.01875 | 1.25E-05|                                                                                         | −0.00114 | 0.7585  |
| cg07393857 | CCDC87; CCS              | 0.013681 | 3.63E-06|                                                                                         | 0.00489  | 0.1686  |
| cg27581091 | GPR123                   | −0.02002 | 5.37E-07|                                                                                         | −0.00805 | 0.1778  |
| cg06843388 | GALNT18                  | −0.04029 | 8.45E-05|                                                                                         | −0.02768 | 0.0185  |
| cg07850274 | RRH                      | 0.014182 | 9.57E-05|                                                                                         | 0.00218  | 0.5863  |
| cg02286547 | WNT11                    | −0.01126 | 0.000127|                                                                                         | −0.00212 | 0.6015  |
| cg06598663 | RH1                      | 0.014224 | 0.00014 |                                                                                         | 0.000577 | 0.8963  |
| cg00919411 | ZBED9                    | 0.018551 | 0.000169|                                                                                         | 0.009982 | 0.0691  |
| cg17305548 | TRAF7                    | −0.0312  | 0.000171|                                                                                         | −0.00573 | 0.1348  |
| cg00806704 | CDX2                     | 0.0139   | 0.000206|                                                                                         | 0.007436 | 0.1086  |
| cg06155414 | MBNL2                    | 0.024019 | 0.000224|                                                                                         | 0.01554  | 0.8297  |
| cg24007886 | OBFC2B; SLC39A5          | 0.021191 | 0.000234|                                                                                         | 0.000316 | 0.9612  |
| cg27583997 | PHF3                     | −0.00661 | 0.00027 |                                                                                         | −0.00225 | 0.2969  |
| cg16532467 |                           | 0.024235 | 0.00027  |                                                                                         | 0.01685  | 0.0243  |
| cg24335950 | PFN1P9                   | −0.01227 | 0.000304 |                                                                                         | −0.00113 | 0.728   |
| cg12152384 | COX4NB; COX4I1           | −0.01524 | 0.000365 |                                                                                         | −0.00311 | 0.5052  |
| cg11535869 |                           | −0.01501 | 0.000449 |                                                                                         | 0.001856 | 0.696   |
| cg23508779 | CDH4                     | 0.00089179 | 0.8495 |                                                                                         | −0.019987255 | 0.00015913 |
| cg24048949 | ZNF556                   | −0.004444 | 0.3776 |                                                                                         | −0.02148841 | 0.00021676 |
| cg00645664 | ABCA13                   | 0.00309189 | 0.4877 |                                                                                         | 0.017962936 | 0.00036261 |

A strength of this study is that two consecutive cohorts were examined, one for discovery and the other for replication. Although based on recruitment, these cohorts are linked to one another, however, due to measurements of DNA methylation and availability of serum samples, the F0-F1 and F1-F2 analyses are widely independent. Only 33 of the 493 F0-F1 mother–offspring pairs were also part of the 183 F1-F2 mother–offspring pairs. Second, we used data on sera metabolite concentrations in the analysis as well as the information collected from questionnaires. Comparing reported acetaminophen use and metabolite clusters showed a sensitivity of 71%, indicating that in 29% acetaminophen metabolites were discovered when the pregnant women did not report using it. However, acetaminophen often is part of combination therapeutics [52–54]. Hence, women may not be aware that they were taking acetaminophen. In the F0, metabolite measurements made possible to associate acetaminophen with DNA methylation for F0-F1 dyads since no reporting of acetaminophen use was sought at the time (1989/90). Thirdly, metabolite level assessment adds important independent information, since only five of 11 CpGs related to reported acetaminophen use were redetected when

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*a* The generalized linear regression models were controlled for covariates including gender, smoking level during pregnancy, asthma, order of birth, CD4T, CD8T, B cells, monocytes, natural killer cells, Neu, and Eos. Bonferroni adjustment was applied on the selected CpG sites.

*b* Bonferroni-adjusted P-value: 0.05/(51 × 2 exposures) = 0.00049.
using metabolite clusters. It is possible that reported use and measured metabolites depict different settings. Reported use may be more directly related to the effect of acetaminophen, whereas the metabolites represent the biological metabolic pathway which may lead to differential effects on DNA methylation. Acetaminophen and its metabolites have short half-lives of only a few hours, so serum levels at childbirth only reflect recent use (estimated as within about 48 h of blood collection), emphasizing the need to consider both reported use and metabolites. Fourth, for both reported use and metabolite clusters it is possible that underlying chronic diseases and actual acetaminophen result in differential DNA methylation. To separate both associations and detect indication biases, we used repeated pregnancy analyses with different settings of chronic diseases and acetaminophen use. About half of the detected CpG sites survived the indication bias analysis.

**Conclusions**

In conclusion, our findings suggest that gestational use of acetaminophen is related to differential DNA methylation in newborn blood. There is a need for additional replication and to investigate whether the reported differential DNA methylation at birth after exposure to acetaminophen can be linked to adverse health outcomes in offspring.

**Data availability**

Phenotypic data are available under the webpage: [http://www.allergyresearch.org.uk/studies/birth-cohort/](http://www.allergyresearch.org.uk/studies/birth-cohort/). Go to “Using our data”. General information on how to access the IOW birth cohort data and the Third Generation Study data is presented in the above webpage. Access to data of the two cohorts must be requested using the formal procedures described and is subject to eligibility, the IOWC funder’s terms and conditions and the Isle of Wight NHS Trust’s policies and procedures. The metabolite data are available MetaboLights (MTBLS4304) at [https://www.ebi.ac.uk/metabolights/editor/www.ebi.ac.uk/metabolights/MTBLS4304](https://www.ebi.ac.uk/metabolights/editor/www.ebi.ac.uk/metabolights/MTBLS4304).

**Supplementary data**

Supplementary data is available at EnvEpig online.

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**Conflict of interest statement**

None declared.

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