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Research Article

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*-benzoquinoneimine (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

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newborn cord blood DNA and can serve as a biomarker of prenatal exposures or as a prognostic marker [21, 23].

Potential mechanisms were identified in experimental studies in rats and human cell cultures, which revealed the effect of acetaminophen on the expression level of important epigenetic machinery components. These experimental studies showed that the expression level of DNA methyltransferases *DNMT3a* and *DNMT3b* decreased following exposure to acetaminophen; however, increased expression level of *TET1*, which plays a role in DNA and histone methylation, was determined by acetaminophen exposure [14, 24]. Nevertheless, changes in genes altering DNA methylation do not explain, why specific CpG sites/genes were differentially methylated after acetaminophen exposure.

We aim to evaluate whether DNAm in newborns is associated with maternal use of acetaminophen during gestation based on maternal information of acetaminophen use and on analyses of acetaminophen and its metabolites in maternal and cord serum. We analyzed data from two consecutive mother–offspring dyads (F0-F1 and F1-F2) from the Isle of Wight (IOW) birth cohort study [25, 26]. For F1-newborns, we have information on acetaminophen metabolites in sera of their F0-mothers collected at birth and DNAm from Guthrie cards in F1. In F2-newborns, available data include 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. Polar metabolites were analyzed 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 ($\Delta m < 3$ 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-d_3) 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 Methylation EPIC 850 K 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 proportions 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

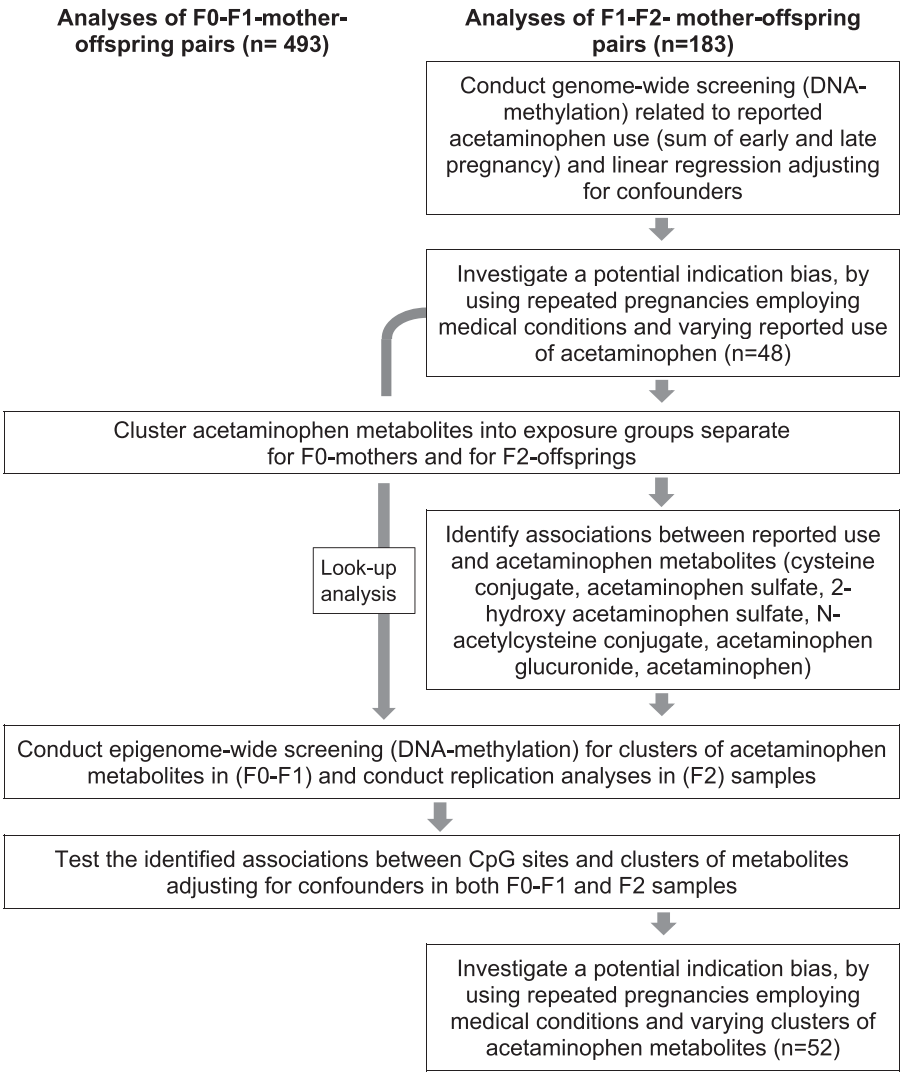

Figure 1: Flow chart of the statistical analyses

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 ttScreening, 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 DNAm 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

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[a]

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[b]	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.
[b]'Winter' was used 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 *CASP5* 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 $\geq 75\%$, 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[a]

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-acetylcysteine 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]. *LINC00589* gene encodes for lncRNAs, 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 ($N = 183$)[a]

Variable		Any acetaminophen use[b]			Acetaminophen use in first trimester			Acetaminophen use in last trimesters		
		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[c]	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	0.75
Season of birth[d]	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	
	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

[a]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' wall. Categorical variables were tested using chi-square goodness of fit test. Continuous variables were tested using one-sample *t*-test.
[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]Infections include bacterial, chest, and/or urinary tract infection based on hospital records.
[d]'Winter' was used as a reference.

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

	Acetaminophen use throughout pregnancy					Acetaminophen use in the first half of the pregnancy					Acetaminophen use in the last half of the pregnancy ($n = 119$)				
	Yes ($n = 51$)		No ($n = 112$)			Yes ($n = 57$)		No ($n = 106$)			Yes ($n = 50$)		No ($n = 69$)		
Metabolites in F2-serum	Median	IQR[a]	Median	IQR[a]	P value	Median	IQR[a]	Median	IQR[a]	P value	Median	IQR[a]	Median	IQR[a]	P value
Acetaminophen sulfate (1.15_231.0197 n)	15.54	4477	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	3.87	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

[a]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 ($n = 48$)

		Early or late prenatal acetaminophen vs. no acetaminophen		Early and late prenatal acetaminophen vs. no acetaminophen	
CpG	UCSC reference gene name	Estimate	*P* value	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	−0.00782	0.0727
cg04944682	ACTR3BP2	0.01904	0.4194	0.03464	0.0931
cg16008631	RPH3AL	−0.00395	0.4104	−0.00749	0.0751
cg26077378	ZNF385D	−0.02434	0.0016	−0.01274	0.0476
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[a]

		Cluster with high metabolite levels except cysteine conjugate ($n = 77$, reference $n = 252$)		Cluster with high levels of all metabolites ($n = 71$, reference $n = 252$)	
CpG	UCSC reference gene name	Estimate	*P* value[b]	Estimate	*P* value[b]
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	2.97E-05	−0.00484	0.351
cg07393857	CCDC87; CCS	0.013681	3.63E-05	0.00489	0.1686
cg27581091	GPR123	−0.02002	6.33E-05	−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	RNH1	0.014224	0.00014	0.000577	0.8963
cg00919411	ZBED9	0.018551	0.000169	0.009982	0.0691
cg17302548	TRAF7	−0.01312	0.000171	−0.00573	0.1348
cg00806704	CDX2	0.0139	0.000206	0.007436	0.1086
cg06155414	MBNL2	0.024019	0.000224	0.001534	0.8297
cg24007886	OBFC2B; SLC39A5	0.021191	0.000234	0.000316	0.9612
cg27583997	PHF3	−0.00661	0.00027	−0.00225	0.2908
cg16532467		0.024235	0.000271	0.016858	0.0243
cg24336590	PFN1P9	−0.01227	0.000304	−0.0013	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	−0.0044441	0.3776	−0.02148841	0.00021676
cg00645664	ABCA13	0.00309189	0.4877	0.017962936	0.00032621

[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.

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 DNAm 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 DNAm 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

Table 7: Association of metabolites clusters with CpGs identified in F1-newborns and replicated in F2-cord blood using linear mixed models for repeated pregnancies and adjusting for confounders[a]

		Cluster with high metabolite levels except cysteine conjugate $n = 31$ (controls $n = 74$)		Cluster with high levels of all metabolites $n = 47$ (controls $n = 74$)	
CpG[b]	UCSC reference gene name	Estimate	*P* value	Estimate	*P* value
cg07850274	RRH	0.0065	0.0378	0.00435	0.1252
cg17302548	TRAF7	−0.0065	0.0244	0.00014	0.9521
cg06155414	MBNL2	−0.01027	0.0625	−0.00240	0.6263
cg12152384	COX4NB; COX4I1	0.006628	0.0588	0.0027	0.3285
cg11535869	UBE3C	0.005861	0.0888	−0.00172	0.5773

[a]The generalized mixed model was controlled for covariates including sex, order of birth, CD4T, CD8T, and B cells. Other cell-types were not confounding.
[b]CpGs that were detected by investigating clusters of metabolites and F1-newborns cord blood DNA methylation levels at CpGs using generalized linear models adjusting for confounders.

Table 8: Association of metabolites clusters with differential methylation of CpG F1-newborns cord blood and the pregnancy control analysis of the same women with the variable underlying conditions and clusters of metabolites ($n = 52$)

		Cluster with high metabolite levels except cysteine conjugate		Cluster with high levels of all metabolites	
CpG[a]	UCSC reference gene name	Estimate	*P* value	Estimate	*P* value
cg02117924	ITGB2	0.01233	0.0237	0.005883	0.2061
cg06155414	MBNL2	−0.02019	0.0352	−0.01193	0.1474

[a]CpGs that were detected by investigating clusters of metabolites and F1-newborns cord blood DNA methylation levels at CpGs using generalized linear models adjusting for confounders.

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 DNAm. 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 DNAm. 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 DNAm in newborn blood. There is a need for additional replication and to investigate whether the reported differential DNAm 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/. 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 [55].

Supplementary data

Supplementary data is available at *EnvEpig* online.

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Conflict of interest statement

None declared.

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