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Prenatal vitamin intake in first month of pregnancy and DNA methylation in cord blood and placenta in two prospective cohorts

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Abstract

Background: Prenatal vitamin use is recommended before and during pregnancies for normal fetal development. Prenatal vitamins do not have a standard formulation, but many contain calcium, folic acid, iodine, iron, omega-3 fatty acids, zinc, and vitamins A, B6, B12, and D, and usually they contain higher concentrations of folic acid and iron than regular multivitamins in the US. Nutrient levels can impact epigenetic factors such as DNA methylation, but relationships between maternal prenatal vitamin use and DNA methylation have been relatively understudied. We examined use of prenatal vitamins in the first month of pregnancy in relation to cord blood and placenta DNA methylation in two prospective pregnancy cohorts: the Early Autism Risk Longitudinal Investigation (EARLI) and Markers of Autism Risk Learning Early Signs (MARBLES) studies.

Results: In placenta, prenatal vitamin intake was marginally associated with -0.52% (95% CI $-1.04, 0.01$) lower mean array-wide DNA methylation in EARLI, and associated with -0.60% ($-1.08, -0.13$) lower mean array-wide DNA methylation in MARBLES. There was little consistency in the associations between prenatal vitamin intake and single DNA methylation site effect estimates across cohorts and tissues, with only a few overlapping sites with correlated effect estimates. However, the single DNA methylation sites with p -value < 0.01 (EARLI cord $n_{\text{CpGs}} = 4068$, EARLI placenta $n_{\text{CpGs}} = 3647$, MARBLES cord $n_{\text{CpGs}} = 4068$, MARBLES placenta $n_{\text{CpGs}} = 9563$) were consistently enriched in neuronal developmental pathways.

Conclusions: Together, our findings suggest that prenatal vitamin intake in the first month of pregnancy may be related to lower placental global DNA methylation and related to DNA methylation in brain-related pathways in both placenta and cord blood.

Keywords: DNA methylation, Prenatal vitamins, Epigenetics, Epidemiology, Pregnancy cohort, Cord blood, Placenta

Introduction

Vitamins and minerals are critical for normal fetal development. The World Health Organization recommends supplementation during pregnancy with iron, folic acid, vitamin A, calcium, and iodine [1]. The American College of Obstetricians and Gynecologists additionally recommends supplementation with choline and vitamins B6, B12, C, and D [2]. Prenatal vitamins do not have a

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standard formulation, but most contain calcium, iodine, omega-3 fatty acids, zinc, and vitamins A and D as well as more iron and B vitamins, and about twice as much folic acid compared to multivitamins [3–6]. In the US, prenatal vitamin use among pregnant people is estimated to be between 78 and 92%, with 55–60% reporting use in the first trimester [7–10]. In another study of the EARLI cohort, 59.7% reported prenatal vitamin use in the first month [6]. Despite the use of these supplements, a recent study found that a significant number of pregnant people in the US still do not meet the recommended nutrient intake levels [11]. Deficiencies in these nutrients are associated with multiple disorders including anemia and preeclampsia in the parent and impaired neurodevelopment, neural tube defects, and recurrent wheezing in the child [4, 12–14]. Understanding the molecular implications of these nutrients on fetal development is critical.

Nutrient levels can impact epigenetic factors such as DNA methylation. Relationships between prenatal vitamin use and DNA methylation have been relatively understudied, as most prior research focused on individual nutrients. For example, folic acid supplementation is associated with DNA methylation differences in cord blood at both differentially methylated regions [15] and differentially methylated positions in an epigenome-wide meta-analysis ($n=1988$) [16]. Another epigenome-wide association study examining vitamin D levels in two cohorts ($n=1416$) found no association with cord blood DNA methylation [17]. Maternal dietary intake of three dietary patterns was not associated with DNA methylation in placental tissue, implying that minor nutritional deficiencies were not associated with differences in DNA methylation ($n=573$) [18]. Conversely, a different study found that prenatal vitamin supplementation was associated with cord blood DNA methylation ($n=130$) [19]. A systematic review of randomized control trials of maternal micronutrient supplementation and DNA methylation in cord blood, blood spots, placental tissue, and buccal swabs found inconsistent results, but these studies varied in exposure timing and dose, types of micronutrients, sample size, and analytical methods [20]. Additional studies of the associations between prenatal vitamin use and DNA methylation are needed.

Cord blood and placental tissues are useful sources of information to understand fetal development. Epigenetic factors such as DNA methylation play a role in normal and abnormal development [21]. DNA methylation patterns from cord blood and placental tissues may be used as biomarkers of in utero exposures or to predict future health [22]. DNA methylation has tissue-specific patterns, and tissues can have varying sensitivity to nutrient changes. Both cord blood and placental tissue develop early in gestation which represents an important window

for early prenatal vitamin supplementation. The umbilical cord begins to form during the fourth week of gestation and blood is flowing in it by the fifth week, but the structure is not fully developed until the 12th week [23]. Umbilical cord blood contains a higher level of hematopoietic stem cells compared to adult blood and includes differentiated cell types such as B cells, natural killer cells, T cells, monocytes, granulocytes, and nucleated red blood cells [24, 25]. The placenta is the site of gas, nutrient, and waste exchange between the parent and fetus and has metabolic and endocrine functions. The trophoblastic cells of the placenta are of fetal origin, but these are in close contact with the maternal decidua and blood vessels through the chorionic villi [26]. The placenta starts to develop from the trophoblast layer of the blastocyst following implantation in the maternal endometrium, around 6–7 days [27]. Following zygote formation epigenetic reprogramming occurs, making early pregnancy a potentially important exposure window for DNA methylation impacts [28]. The associations between prenatal vitamin use in the first month of pregnancy and DNA methylation in cord blood and placental tissue are still unclear. Here, we examined use of prenatal vitamin use during the first month of pregnancy and DNA methylation in cord blood and placenta in two prospective pregnancy cohorts. We analyzed array-wide mean DNA methylation, as well as associations at single DNA methylation sites and tested for enriched gene ontology processes.

Results

Study sample descriptive statistics

For cord blood, variables of interest (prenatal vitamin use, maternal education, maternal age, genetic principal components, sex, gestational age, estimated cell proportions) were available for 113 samples (66.5% of total) in EARLI and 201 samples (82.7% of total) in MARBLES. For placenta, variables of interest were available for 88 samples (69.3% of total) in EARLI and 70 samples (77.8% of total) in MARBLES. Analyses were done separately in the four cohort/tissue groups: EARLI cord blood, EARLI placenta, MARBLES cord blood, and MARBLES placenta. In all cohort/tissue groups, DNA methylation principal components were associated with sex and estimated cell proportions ($p < 0.001$ in related to at least one of principal components one through three). Laboratory batch was associated with DNA methylation principal components in EARLI cord ($p = 0.002$ with principal component 2) and MARBLES cord ($p = 0.002$ with principal component 2, $p < 0.001$ with principal component 5) (Additional file 1: Figure S3). In cord blood, cell type composition of monocytes ($p < 0.001$) and nRBC ($p < 0.001$) differed between cohorts. In placenta, composition estimates of

Hofbauer cells ($p < 0.001$) and nRBC ($p = 0.003$) differed between cohorts.

Among subjects with cord blood samples, use of prenatal vitamins in the first month of pregnancy was associated with maternal education in both cohorts. In EARLI, 69.0% of mothers who took prenatal vitamins in the first month had a college degree, compared to 42.9% of mothers who did not take prenatal vitamins. In MARBLES, 63.7% of mothers who took prenatal vitamins in the first month of pregnancy had at least a college degree, while

34.3% of mothers who did not take prenatal vitamins had a college degree (Table 1). Among the subset with placenta samples, this same trend was less pronounced in EARLI (64.2% mothers who took prenatal vitamins had college degree versus 51.4% among mothers who did not take prenatal vitamins), and present in MARBLES (69.7% taking prenatal vitamins in first month had college degree compared to 43.2% among those without prenatal vitamin use) (Table 2). By the latter half of pregnancy, the vast majority of mothers (>75%) were taking prenatal

Table 1 Distributions of maternal and fetal participant and sample characteristics for those with cord blood DNA methylation measures, split by maternal use of prenatal vitamins in month 1 of pregnancy

	MARBLES			EARLI		
	No use of prenatal vitamins month 1	Use of prenatal vitamins month 1	P-value	No use of prenatal vitamins month 1	Use of prenatal vitamins month 1	P-value
	N = 99	N = 102		N = 42	N = 71	
Maternal education						
No degree	65 (65.7%)	37 (36.3%)	<0.001	24 (57.1%)	22 (31.0%)	0.01
College degree	34 (34.3%)	65 (63.7%)		18 (42.9%)	49 (69.0%)	
Maternal age at delivery (years)	34.8 (4.95)	34.0 (4.67)	0.26	33.7 (4.48)	33.1 (4.46)	0.51
Maternal pre-pregnancy BMI	27.5 (6.92)	27.9 (6.89)	0.67	27.6 (6.54)	27.6 (7.89)	0.98
Child race/ethnicity						
Non-Hispanic White	41 (41.4%)	44 (43.1%)	0.63	22 (52.4%)	36 (50.7%)	1.00
Hispanic	35 (35.4%)	30 (29.4%)		5 (11.9%)	9 (12.7%)	
Other/multiple	23 (23.2%)	28 (27.5%)		15 (35.7%)	26 (36.6%)	
Child sex						
Female	37 (37.4%)	43 (42.2%)	0.58	25 (59.5%)	29 (40.8%)	0.08
Male	62 (62.6%)	59 (57.8%)		17 (40.5%)	42 (59.2%)	
Gestational age at delivery (weeks)	39.2 (1.15)	39.1 (1.27)	0.52	39.1 (1.49)	39.5 (1.33)	0.24
Estimated cell proportion						
CD8T	4.11 (2.89)	3.66 (2.75)	0.26	3.51 (2.55)	3.99 (3.00)	0.37
CD4T	17.4 (6.08)	17.8 (5.66)	0.66	17.6 (8.36)	17.1 (7.31)	0.74
Natural killer	3.25 (2.90)	3.05 (2.73)	0.62	3.39 (2.48)	3.71 (2.93)	0.53
B cell	5.62 (2.88)	5.36 (2.36)	0.49	5.57 (2.75)	5.68 (3.10)	0.84
Monocytes	10.1 (3.58)	9.55 (2.58)	0.20	6.92 (3.20)	7.38 (2.88)	0.44
Granulocytes	54.7 (11.8)	55.3 (9.29)	0.68	56.7 (13.0)	56.5 (12.0)	0.95
Nucleated RBC	5.04 (6.90)	5.49 (5.88)	0.62	8.02 (6.81)	6.87 (4.92)	0.34
Mean DNA methylation						
Overall	60.9 (0.91)	60.9 (0.86)	0.56	51.0 (0.63)	50.9 (0.98)	0.23
Open sea	76.7 (1.15)	76.6 (1.11)	0.64	74.0 (0.80)	73.7 (1.33)	0.17
CpG shelf	78.1 (1.07)	78.0 (1.01)	0.69	78.4 (0.88)	78.2 (1.46)	0.21
CpG shore	49.6 (0.83)	49.5 (0.72)	0.58	47.0 (0.65)	46.8 (0.91)	0.17
CpG island	18.0 (0.46)	17.9 (0.27)	0.12	19.5 (0.53)	19.4 (0.66)	0.80
DNA methylation laboratory batch ¹						
1	33 (33.3%)	33 (32.4%)	0.89	38 (90.5%)	50 (70.4%)	0.03
2	37 (37.4%)	36 (35.3%)		4 (9.52%)	21 (29.6%)	
3	29 (29.3%)	33 (32.4%)				

¹ The EARLI and MARBLES samples were run separately. In MARBLES cord batch represents sample plate, in EARLI cord batch represents two different runs.

Table 2 Distributions of maternal and fetal participant and sample characteristics for those with placenta DNA methylation measures, split by maternal use of prenatal vitamins in month 1 of pregnancy

	MARBLES			EARLI		
	No use of prenatal vitamins month 1	Use of prenatal vitamins month 1	P-value	No use of prenatal vitamins month 1	Use of prenatal vitamins month 1	P-value
	N = 37	N = 33		N = 35	N = 53	
Maternal education						
No degree	21 (56.8%)	10 (30.3%)	0.05	17 (48.6%)	19 (35.8%)	0.33
College degree	16 (43.2%)	23 (69.7%)		18 (51.4%)	34 (64.2%)	
Maternal age at delivery (years)	35.3 (5.16)	35.1 (5.86)	0.89	33.8 (4.46)	33.5 (4.75)	0.77
Maternal pre-pregnancy BMI	29.0 (8.21)	26.4 (5.86)	0.12	26.8 (5.90)	27.9 (8.20)	0.47
Child race/ethnicity						
Non-Hispanic White	16 (43.2%)	8 (24.2%)	0.11	16 (45.7%)	25 (47.2%)	0.34
Hispanic	14 (37.8%)	12 (36.4%)		5 (14.3%)	11 (20.8%)	
Other/multiple	7 (18.9%)	13 (39.4%)		14 (40.0%)	17 (32.1%)	
Child sex						
Female	17 (45.9%)	7 (21.2%)	0.05	18 (51.4%)	17 (32.1%)	0.11
Male	20 (54.1%)	26 (78.8%)		17 (48.6%)	36 (67.9%)	
Gestational age at delivery (weeks)	39.4 (1.14)	39.2 (0.83)	0.49	39.1 (1.59)	39.4 (1.22)	0.39
Estimated cell proportion						
Trophoblasts	14.0 (7.55)	15.2 (6.14)	0.47	13.0 (6.04)	14.4 (5.13)	0.27
Stromal	9.06 (6.41)	10.0 (6.26)	0.52	10.3 (3.75)	9.47 (3.51)	0.31
Hofbauer	4.71 (2.22)	4.24 (2.22)	0.37	2.73 (1.45)	2.77 (1.54)	0.89
Endothelial	5.43 (2.22)	7.25 (3.68)	0.02	5.57 (2.45)	6.05 (2.63)	0.38
nRBC	0.80 (1.05)	0.55 (1.02)	0.32	1.24 (1.00)	1.46 (2.11)	0.51
Syncytiotrophoblast	66.0 (12.6)	62.8 (9.74)	0.23	67.1 (9.18)	65.9 (8.63)	0.51
Mean DNA methylation						
Overall	53.3 (1.09)	52.8 (1.12)	0.05	45.0 (1.04)	44.5 (1.15)	0.03
Open sea	65.3 (1.41)	64.7 (1.49)	0.06	62.1 (1.17)	61.6 (1.48)	0.09
CpG shelf	69.2 (1.26)	68.5 (1.50)	0.06	68.4 (1.29)	67.8 (1.61)	0.11
CpG shore	45.5 (1.12)	45.0 (1.09)	0.06	42.7 (1.17)	42.1 (1.14)	0.02
CpG island	18.7 (0.69)	18.6 (0.70)	0.64	20.1 (1.00)	19.6 (0.92)	0.02
DNA methylation laboratory batch ¹						
1	–	–	–	32 (91.4%)	46 (86.8%)	0.73
2				3 (8.57%)	7 (13.2%)	

¹ The EARLI and MARBLES samples were run separately. In EARLI placenta batch represents two different runs, MARBLES placenta were measured in a single run on single plate

vitamins (Additional file 1: Table S1). Estimated cell type did not differ by prenatal vitamin status, and maternal pre-pregnancy BMI did not differ but prenatal vitamin use.

Mean DNA methylation differences with prenatal vitamin use

Mean array-wide DNA methylation differed by prenatal vitamin use in the first month of pregnancy in placenta (Additional file 1: Figure S4). In EARLI, prenatal vitamin use was marginally associated with -0.52% (95% confidence interval: $-1.04, 0.01$) lower mean array-wide

placenta DNA methylation. In MARBLES, prenatal vitamin use in the first month was associated with -0.60% (95% confidence interval: $-1.08, -0.13$) lower mean array-wide placenta DNA methylation. Differences by prenatal vitamin use were smaller in magnitude for island regions. In cord blood there were no differences in mean array-wide DNA methylation by prenatal vitamin use in either cohort.

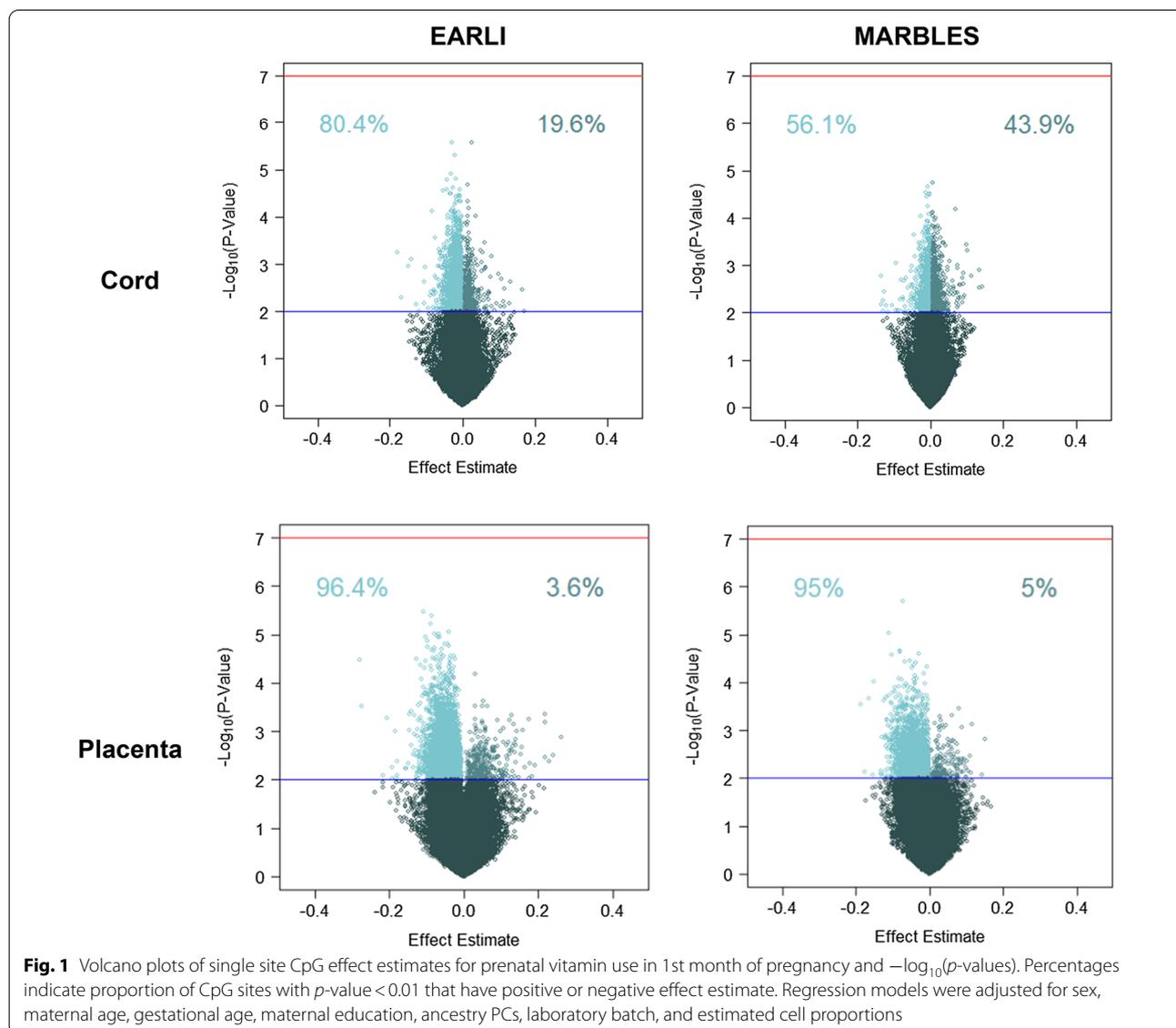
Prenatal vitamin epigenome-wide association results

Across all analyses, no single CpG site met the more stringent significance threshold (p -value $< 10^{-7}$). In

EARLI placenta tissue 3442 sites had a nominal association (p -value < 0.01). Of these sites, 94.4% had lower DNA methylation with prenatal vitamin use in the first pregnancy month and these sites had an average of -3.9 percent lower DNA methylation (Fig. 1). The top site associated with prenatal vitamin use in placenta in EARLI was cg24700222 associated with *COL24A1* gene (effect estimate = -7.3, p -value = 2.0×10^{-6}) (Additional file 2: Table S2). In MARBLES placental tissue, 9216 sites had p -value < 0.01, and 96.4% had lower DNA methylation with prenatal vitamin use and these sites had an average of -4.2 percent lower DNA methylation. The top site associated with prenatal vitamin use in placenta in MARBLES was cg00711959 (effect estimate = -11.0, p -value = 3.3×10^{-6}), which is located on chromosome

2 and is not annotated to a gene (Additional file 3: Table S3).

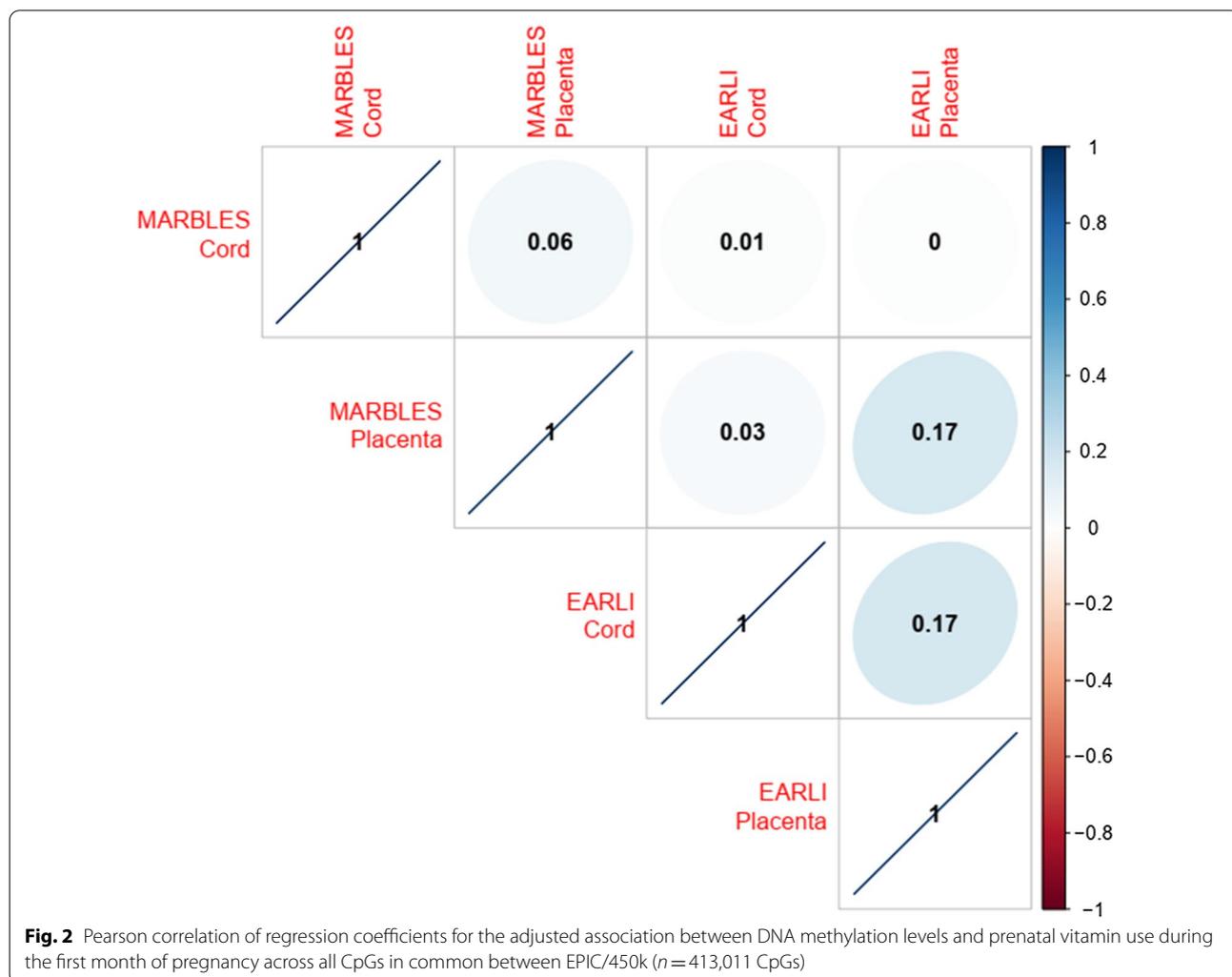
In cord blood, among nominally 3273 associated sites in EARLI, 80.5% had lower DNA methylation with prenatal vitamin use and these sites had an average of 1.8 percent lower DNA methylation. The top site associated with prenatal vitamin use in cord blood in EARLI was cg18452703 (effect estimate = -3.0, p -value = 2.7×10^{-6}), located on chromosome 10 and not annotated to a gene. (Additional file 4: Table S4). In MARBLES cord blood, 58.3% of the 2,348 nominally associated sites had lower DNA methylation for prenatal vitamin use and these sites had an average 1.0 percent lower DNA methylation. The top site associated with prenatal vitamin use in cord blood in

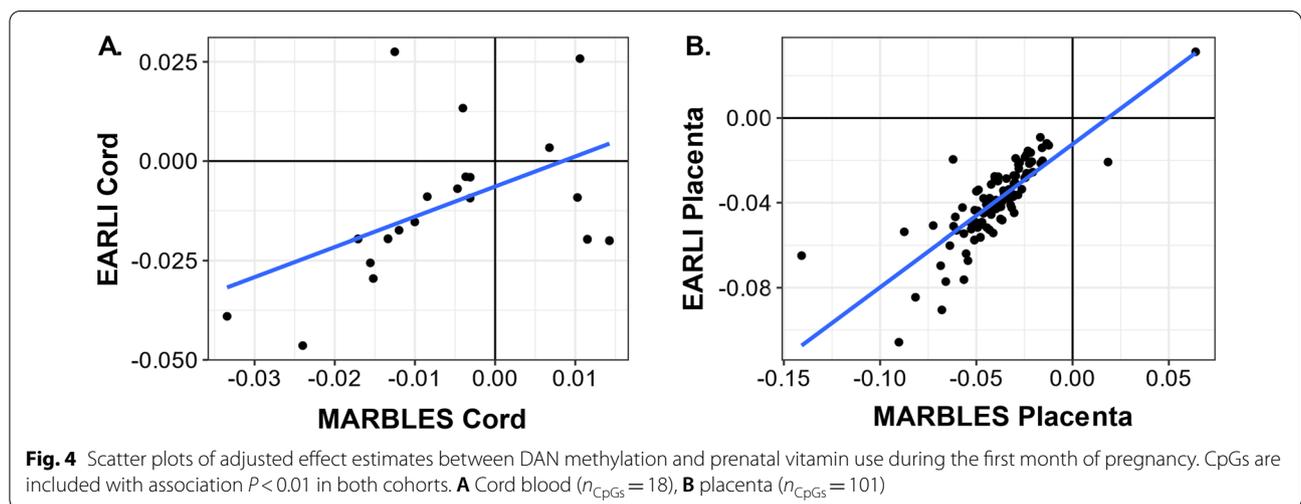
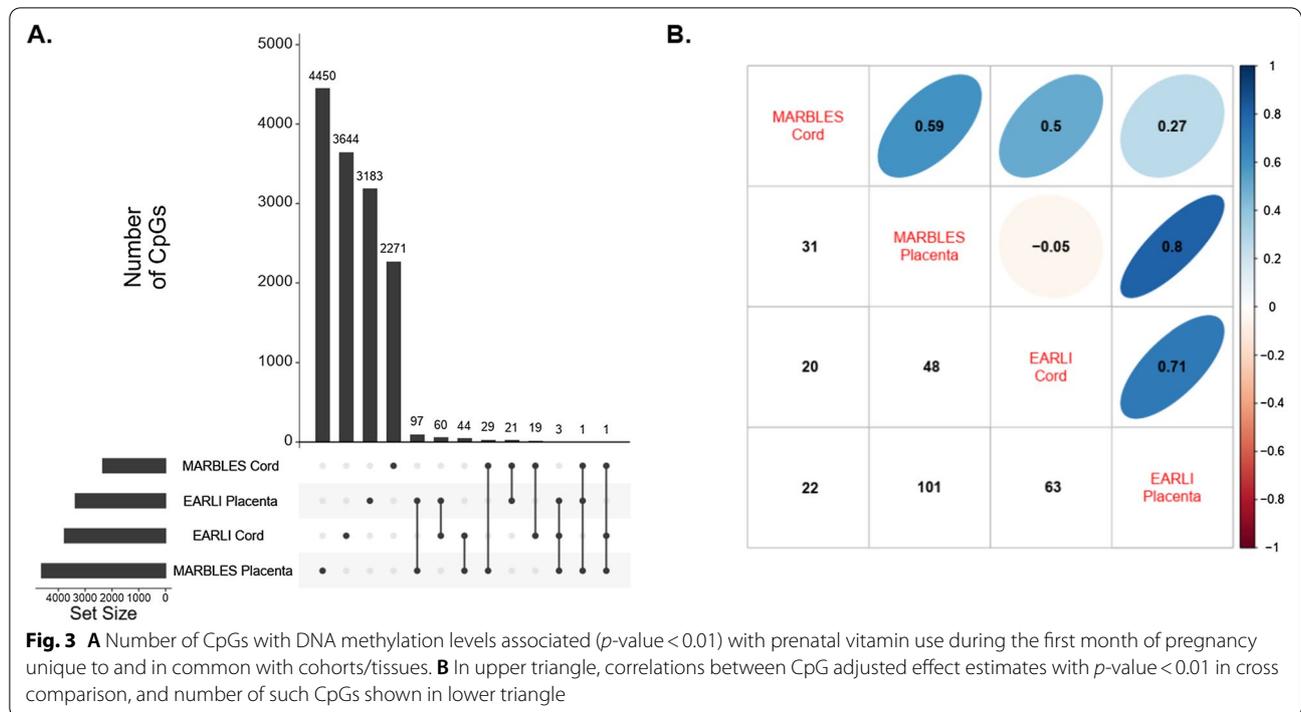


MARBLES was cg04551619 (effect estimate=0.56, p -value = 1.8×10^{-9}), which is located on chromosome 1 and is not annotated to a gene (Additional file 5: Table S5).

For the DNA methylation sites commonly measured across tissues and cohorts ($n=422,383$ CpGs), overall correlations among effect estimates for the associations of prenatal vitamin use and DNA methylation were weak to modest (Fig. 2). The strongest correlations in effect estimates were between MARBLES placenta (measured on EPIC) and EARLI placenta (measured on 450k) ($r=0.17$, p -value $< 2.2 \times 10^{-16}$), and between EARLI cord (measured on 450k) and EARLI placenta ($r=0.17$, p -value $< 2.2 \times 10^{-16}$). In contrast, correlation of cord blood effect estimates between the cohorts was non-existent ($r=0.02$, p -value $< 2.2 \times 10^{-16}$), and correlation between cord blood and placenta in MARBLES was similarly weak ($r=0.06$, p -value $< 2.2 \times 10^{-16}$).

We restricted to the DNA methylation sites associated (p -value < 0.01) with prenatal vitamin use and calculated overlap and correlation across tissues and cohorts. In EARLI cord blood, 4068 CpGs had p -value < 0.01 , EARLI placenta had 3647 CpG sites, MARBLES cord had 4,025 CpGs, and MARBLES placenta had 9563 CpGs. Sites reaching this threshold were largely tissue and cohort specific (Fig. 3A). The largest levels of overlap mirrored the correlation patterns. Between EARLI and MARBLES placenta, 101 CpG sites had p -value < 0.01 , and these 101 sites had correlation $r=0.80$ (Fig. 3B). The 101 CpG sites had effect estimates in the same direction in MARBLES and EARLI placenta, except for one site, with 99 of them having negative effect estimates (Fig. 4). In contrast, comparing cord blood between MARBLES and EARLI there were 20 CpGs with p -value < 0.01 in both (Fig. 3B), with more modest correlation ($r=0.50$) and mixed directions of effect (Fig. 4). Between cord blood and placenta in EARLI, 63 sites had p -value < 0.01 in both, with





those CpG effect estimates having correlation $r = 0.71$. In MARBLES, 31 CpGs had p -value < 0.01 in both cord blood and placenta, with effect estimates having correlation $r = 0.59$ (Fig. 3B). EARLI had more consistency in direction of effect between cord blood and placenta than MARBLES (Additional file 1: Figure S5).

We compared single site results with a previous study on maternal plasma folate during pregnancy and cord blood methylation (data were accessed through dbGaP, accession number: phs001059.v1.p1.c1) [16]. In that

study, 7219 CpG sites had p -value < 0.01 . Looking only at sites overlapping between the 450k and EPIC chips, the number of sites with p -value < 0.01 was 3365 in EARLI placenta, 3771 in EARLI cord, 4625 in MARBLES placenta, and 2342 in MARBLES cord. The level of overlap was minimal but more than expected at random between the maternal plasma folate study and our prenatal vitamin usage study, with 72 CpGs overlapping with EARLI placenta results (Fisher test $p = 0.007$), 131 CpGs with EARLI cord (Fisher test $p < 0.001$), 96 CpGs with

MARBLES placenta (Fisher test $p=0.009$), and 45 CpGs with MARBLES cord (Fisher test $p=0.10$).

Gene ontologies enriched in prenatal vitamin use-related DNA methylation

CpG sites associated with prenatal vitamin use (p -value < 0.01) in the epigenome-wide regression models were used for gene ontology analysis (EARLI cord $n_{CpGs}=4068$, EARLI placenta $n_{CpGs}=3647$, MARBLES cord $n_{CpGs}=4025$, MARBLES placenta $n_{CpGs}=9563$). Across all four tissue and cohort combinations, the top pathways by rank sum were neuron and development-related pathways, such as nervous system development, neuron differentiation, and cell projection morphogenesis (Table 3, Additional file 6: Table S6). In cord blood, the top pathways by rank sum across the two cohorts included largely development-related pathways, such as sensory organ development, neuron differentiation, and embryo development. In MARBLES cord blood, no pathways had $FDR < 1.0$, and the top pathway was protein catabolic process in the vacuole (Additional file 7: Table S7). In EARLI cord blood, no pathways had $FDR < 1.0$, and the top pathway was nervous system development (Additional file 8: Table S8). In placenta, the top pathways by rank sum across the two cohorts were neuron and synaptic signaling pathways, including neurogenesis, and

chemical synaptic transmission. In MARBLES placenta, the top pathway was neuron development ($FDR=0.14$) (Additional file 9: Table S9). In EARLI placenta, the top pathways were regulation of bone remodeling ($FDR=0.48$) and system development ($FDR=0.48$) (Additional file 10: Table S10). Comparing the pathways with unadjusted p -value < 0.01, mirroring single site comparisons most pathways were unique to tissue/cohort (Additional file 1: Figure S6). A total of eight pathways, including neuron differentiation and chemical synaptic transmission, had $p < 0.01$ in all but MARBLES cord blood.

The top 1000 CpG sites associated with prenatal vitamin use in the first month of pregnancy were examined for enrichment in chromatin state marker signatures. Sites associated with prenatal vitamin use in EARLI cord blood were enriched in the repressed polycomb chromatin state, followed by weak repressed polycomb, bivalent enhancer, and bivalent/poised transcript start sequence markers (Additional file 1: Figure S7).

Replication testing with whole genome bisulfite sequencing (WGBS)

Data for WGBS were available on 63 samples [33 with prenatal vitamin use] in EARLI cord blood, 91 samples [39 with prenatal vitamin use] in MARBLES placenta,

Table 3 Top 20 biologic pathways by rank sum across both cohorts and tissues

Biologic pathway	MARBLES cord rank	MARBLES placenta rank	EARLI cord rank	EARLI placenta rank	Rank sum
Behavior	33	29	41	137	240
Nervous system development	718	8	1	8	735
Axon development	255	282	368	13	918
Regulation of synaptic plasticity	116	313	122	434	985
Synapse organization	760	25	285	42	1112
Neurogenesis	1125	5	18	32	1180
Cell morphogenesis involved in differentiation	424	35	671	121	1251
Neuron differentiation	1218	3	17	39	1277
Axonogenesis	149	270	884	11	1314
Neuron development	1257	1	178	35	1471
Central nervous system development	742	67	348	320	1477
Eating behavior	1069	162	104	260	1595
Cognition	30	146	197	1286	1659
Cell morphogenesis involved in neuron differentiation	663	54	975	46	1738
Generation of neurons	1726	10	16	23	1775
Import into cell	165	394	513	767	1839
Neuron recognition	56	716	686	443	1901
Long-term synaptic potentiation	282	363	869	408	1922
Learning or memory	54	334	214	1376	1978
Anatomical structure morphogenesis	1163	471	175	169	1978

Pathway results from missMethyl, using CpG sites associated with prenatal vitamin use in month 1 of pregnancy with $P < 0.01$ as input (MARBLES cord $n_{CpGs}=4455$, MARBLES placenta $n_{CpGs}=9340$, EARLI cord $n_{CpGs}=4032$, EARLI placenta $n_{CpGs}=3589$)

45 samples [17 with prenatal vitamin use] in MARBLES cord on HiSeq 4000, and 42 samples [17 with prenatal vitamin use] in MARBLES cord on HiSeq X Ten. Differentially methylated regions (DMRs) with p -value < 0.05 in each are summarized in Additional file 11: Table S11. Overall, a majority of DMRs showed lower DNA methylation with prenatal vitamin use, and agreement in the direction of effect was strongest in placenta, where there were 803 array sites within 5 kb of DMRs identified in WGBS, and 66.6% had the same direction of effect across the two measures (Additional file 1: Table S12).

Discussion

In two prospective pregnancy cohorts, we found that prenatal vitamin intake in the first month of pregnancy was related to lower average DNA methylation in placenta and cord blood. The magnitude of this association was strongest in placenta. We observed little consistency in the associations between prenatal vitamin intake and single DNA methylation site effect estimates across cohorts and tissues, with only a few overlapping sites with correlated effect estimates. However, the single DNA methylation sites associated with prenatal vitamin use were consistently enriched in neuron developmental pathways. Together these findings suggest that prenatal vitamin intake may be related to placental global DNA methylation and related to DNA methylation in brain-related pathways in both placenta and cord blood.

Previous research has examined DNA methylation and prenatal vitamin supplementation, or components of prenatal multivitamins, at different supplementation time points. Vitamin B12 is a component of prenatal vitamins, and prenatal deficiency leads to adverse infant hematologic and neurologic outcomes [29]. In terms of DNA methylation, a cohort study of cord blood samples found that higher maternal serum vitamin B12 concentrations (collected at mean 10.6 weeks of gestation) were associated with lower global cord blood DNA methylation, using bisulfite pyrosequencing [30]. Another study of 516 cord blood samples observed periconceptional vitamin B12 intake was not associated with long interspersed nuclear element-1 (LINE-1) DNA methylation, often used as a proxy for global methylation [31]. Placentas from rats who were treated with excess folate and were vitamin B12 deficient had lower global DNA methylation levels compared to controls and rats with normal folate + B12 deficiency, but when they were given omega-3, the DNA methylation returned to the control group level [32]. In humans, mothers with high folate and low vitamin B12 during early pregnancy had significantly lower cord blood DNA methylation as measured by LC-MS/MS [33]. These previous studies were heterogeneous in

species, sample type, and DNA methylation measurement methods. The conflicting results highlight the need for more comprehensive analyses of the associations between early pregnancy vitamin supplementation and DNA methylation, considering the intake of multiple vitamins.

Some prenatal vitamins also contain omega-3 fatty acids. The omega-3 fatty acid, docosahexaenoic acid (DHA) and polyunsaturated fatty acids are essential during pregnancy for fetal development [34]. A study of DHA, given at 20+ weeks of gestation, found no difference in global DNA methylation of blood spots and leukocytes between the children from the treatment and control groups [35]. They did identify 21 differentially methylated regions with most regions showing lower methylation levels in the treatment group compared to the control. The direction of this finding matches ours where a majority of our differentially methylated sites (p -value < 0.01) across both cohorts and tissue types associated lower methylation with prenatal vitamin use. A randomized control trial of polyunsaturated fatty acid supplementation from weeks 18–22 to birth observed no difference in the cord blood global DNA methylation between the non-smoking treatment and control groups [36]. The review by Andraos et al. (2018) summarizes the DNA methylation results from randomized control trials of nutritional supplementations during pregnancy, finding overall that micronutrient supplementation does not substantially affect offspring DNA methylation in cord blood, blood spots, placental tissue, and buccal swabs, though lack of standardized methods complicate comparing results of studies [20].

Folate and folic acid are components of prenatal vitamins. In a randomized control trial of maternal folic acid supplementation in the second and third trimesters, supplementation was associated with lower DNA methylation in cord blood at LINE-1 compared to the control group [37, 38]. A meta-analysis of two cohorts of 1,988 infants identified 443 DNA methylation sites in cord blood associated (FDR < 0.05) with maternal plasma folate levels measured during pregnancy (median 18 weeks gestation, and median 12.9 weeks gestation in the two cohorts) [16]. Of these sites, 416 (94%) had lower DNA methylation with higher plasma folate levels, suggesting a similar direction of association to what we observed. Using gene ontologies, prenatal vitamin-associated DNA methylation sites were enriched for developmental and neuronal pathways in both our cohorts and tissues. The meta-analysis also observed an enrichment of developmental and neurodevelopmental pathways [16]. Although we did not observe correlation of associations at individual DNA methylation sites to this previous study, our findings were consistent with respect to

overall trends of lower DNA methylation and pathway enrichment.

Prenatal vitamins overlap with multivitamins in their nutrient components, although prenatal vitamins typically contain more folic acid than multivitamins (about twice as much) [5]. Previously in the EARLI cohort, multivitamin usage in the 3 months before pregnancy in mothers without the *MTHFR* allele variant was associated with higher global cord blood DNA methylation [19]. However, similar to our current findings in cord blood, the previous EARLI study observed no association between prenatal vitamin use in the 3 months prior to pregnancy and global cord blood DNA methylation ($p < 0.05$). These analyses differed in their exposure window (prior paper: 3 months prior to pregnancy versus current paper: first month of pregnancy).

Differences in DNA methylation have been associated with other early-life exposures to environmental factors, in addition to prenatal vitamin use [39–41]. In this study, we found larger differences in DNA methylation and more consistency between cohorts in placenta compared to cord blood. This finding is likely due to known DNA methylation characteristics of placenta, including global hypomethylation relative to somatic tissues and the presence of partially methylated domains [42]. In placenta, methylation of gene bodies is predictive of active expression and exposure to home/garden pesticides was previously shown to be associated with higher global DNA methylation in MARBLES placenta samples [43, 44]. Combined, these findings suggest that the lower global DNA methylation levels associated with prenatal vitamin use may be reflecting a more quiescent genome with reduced activation of environmentally responsive genes that may be related to the known effects of nutrients in reducing oxidative stress [45]. We observed surprisingly low correlation in cord blood effect estimates between the two cohorts. This could suggest a lack of robust single site effects, but batch effects may have contributed to reduced correlation. Within the EARLI study, we observed higher correlation between the placenta and cord blood DNA methylation associations with prenatal vitamin intake, than we did in the MARBLES study. The EARLI placenta and cord blood DNA methylation measures were conducted at the same time and on the same laboratory plates. In MARBLES, the cord blood and placenta samples were run at different times and on different laboratory plates. Cell type proportions are a large driver of variability in DNA methylation measures in most tissues. Environmental exposures can even be highly associated with cell type proportions [46], and cell type proportions can mediate the effects of environmental exposures on DNA methylation [47]. In our study, cell composition did not differ by prenatal vitamin status, but

we did observe differences in the cell composition of tissues by cohort.

The prior studies reviewed above and the current study have great heterogeneity with respect to the type, method, and timing of prenatal vitamin-related exposure measures. As described, the associations vary, but more studies have reported lower DNA methylation with pregnancy vitamin use. Most environmental epigenetic studies have used the Illumina BeadArrays for DNA methylation measures, though the tissue measured at birth varies. Replication testing or meta-analyses are necessary to determine the reproducibility of these findings and few studies to date have included more than one cohort. These steps will require harmonization of exposure and DNA methylation measures across studies. Once reproducibility and specificity are determined, these DNA methylation patterns may be used as biomarkers of exposure or may predict future health [22].

There were a number of strengths to this study. We analyzed two prospective pregnancy cohorts and two tissue types with 201 total samples from EARLI and 271 total samples from MARBLES. The prospective design minimized recall bias of prenatal vitamin use. Furthermore, we had consistent exposure measures including using data from questionnaires, consistent cleaning of the vitamin questionnaires, and consistent exposure timing during pregnancy. The results were based on a diverse subset of participants from five study sites in the US. We employed rigorous methods to preprocess and analyze the DNA methylation data. Our analysis across the two cohorts and two tissue types showed similar gene ontology pathways. Finally, we compared the array-based DNA methylation single site results to the WGBS results.

There were several limitations to our study. First, the samples used for DNA methylation were from one time point at birth, so long-term differences in DNA methylation were not assessed. We examined prenatal vitamin use as a yes/no response for any use in the first pregnancy month; effects may differ by frequency of intake, nutrient composition, and nutrient dose. We did not account for differences in underlying nutritional deficiencies which may have affected our results. Future studies could assess nutritional intake in addition to prenatal vitamins. Exposure to other environmental chemicals may lower the DNA methylation levels of adults, children, and infants [30, 48–52]. It is possible that differences in chemical exposures may account for or mask some of the differences across DNA methylation related to prenatal vitamin use. While there was little positional overlap in effects between array-based DNA methylation and WGBS, we observed similar directions of effect across measurement methods in placenta. This finding may have been due to a greater overlap in these samples

between measured on array compared to WGBS. Data on method of delivery, known to affect DNA methylation [53], were incomplete in EARLI, so we did not adjust for that information in our regression analyses for both cohorts. Additional caution should be applied when interpreting our findings, since prenatal vitamin use was associated with higher educational attainment, and there may be unmeasured confounding with such factors as improved prenatal care, improved diet, and other factors. Studies with available measures in these areas will be highly valuable. Future studies could examine a larger cohort, compare these results to a general population cohort, or conduct a meta-analysis of multiple prenatal vitamin studies. Future studies may also consider examining the placenta, to explore whether it is more sensitive to effects of supplementation, as our study suggests.

Conclusions

We found that prenatal vitamin use in the first month of pregnancy was associated with lower DNA methylation, particularly in the placenta. Prenatal vitamin use is recommended before and during pregnancies for normal fetal development. Given its importance, additional research is needed to understand the underlying biological mechanisms of development. By demonstrating an association between prenatal vitamin intake and DNA methylation at birth, we lay the foundation for DNA methylation as a biomarker of prenatal vitamin exposure. While promising, these findings also highlight the need for larger studies in this area with standardized, well measured, and longitudinal prenatal vitamin exposure measures for testing with epigenetic marks and fetal and postnatal growth and health.

Methods

Study samples

The Early Autism Risk Longitudinal Investigation (EARLI) and Markers of Autism Risk Learning Early Signs (MARBLES) studies are enriched risk prospective pregnancy cohorts studying autism etiology [54, 55]. The EARLI study was reviewed and approved by Human Subjects Institutional Review Boards (IRBs) from each of the four study sites (Johns Hopkins University, Drexel University, University of California Davis, and Kaiser Permanente Northern California). The MARBLES protocol was reviewed and approved by the Human Subjects IRB from University of California Davis. Secondary data analysis for this manuscript were approved by the Human Subjects IRB for the University of Michigan. Both studies recruited mothers of children with clinically confirmed ASD who were early in a subsequent pregnancy or were trying to become pregnant. In EARLI there were 232 mothers with a subsequent sibling born through

this study between November 2009 and March 2012. In MARBLES there were 389 enrolled mothers that gave birth to 425 subsequent siblings between December 1, 2006 and July 1, 2016.

Covariate and exposure assessment

Demographics, behaviors, and medical history were all collected longitudinally via maternal self-report questionnaire. In these questionnaires, mothers were asked if they used prenatal vitamins for each month of pregnancy (yes/no). Data for the first month of pregnancy and for 3 months prior to pregnancy were collected at study enrollment.

Sample collection and processing

In EARLI, biospecimens including cord blood and placenta, were collected and archived for 213 births. Full thickness placental tissue from a central cotyledon was collected. Sterile punch biopsy forceps were used to extract placental samples from the maternal and fetal sides. Whole cord blood was also collected at delivery. Samples were transported to the Johns Hopkins Biological Repository (JHBR) for aliquoting and archiving (-80°C). Placental DNA was extracted with the DNeasy Tissue Kit (Qiagen), and cord blood DNA was extracted using the DNA Midi kit (Qiagen, Valencia, CA). DNA was quantified using the Nanodrop (ThermoFisher Waltham, MA) and normalized DNA aliquots were sent to the Center for Inherited Disease Research (Johns Hopkins University). DNA samples were bisulfite treated and cleaned using the EZ DNA methylation gold kit (Zymo Research, Irvine, CA) according to manufacturer's instructions. DNA was plated randomly and was assayed on the Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA) [56]. Methylation control gradients and between-plate repeated tissue controls were used.

In MARBLES, placental tissues and cord blood were collected at delivery and immediately processed and frozen. The MARBLES study used orientation to the umbilical cord to ensure that all placenta samples were isolated from the chorionic villus from the fetal side of the placenta. Placental and cord blood samples were stored at -80°C in the UC Davis repository. Cord blood and placenta samples were processed for methylation measures. Placenta DNA was extracted with Gentra Puregene kit (Qiagen) and cord blood DNA was extracted using the DNA Midi kit (Qiagen, Valencia, CA). Samples were bisulfite treated and cleaned using the EZ DNA methylation gold kit (Zymo Research, Irvine, CA). DNA was plated randomly and assayed on the Infinium HumanMethylationEPIC BeadChip (Illumina, San Diego, CA) at the Johns Hopkins SNP Center, a shared lab and informatics operation with the Center for Inherited Disease

Research (Johns Hopkins University). DNA methylation control gradients and between-plate repeated tissue controls were used.

DNA methylation processing

For all methylation samples, we used the minfi library (version 1.30.0) in R (version 3.6) to process raw Illumina image files into noob background corrected methylation values [57, 58]. In EARLI, cord blood and placenta samples were run on the 450k array together in two batches, and thus preprocessed together. Samples from multiple births (cord blood $n=2$ samples, placenta $n=6$ samples), as well as samples with discordant DNA methylation predicted sex and observed infant sex were removed (cord blood $n=3$, placenta $n=1$). Probes with failed detection P -value (>0.01) in $>5\%$ of samples were removed ($n=661$), as were probes documented as cross-reactive ($n=29,153$) [59]. Y-chromosome probes ($n=48$) were dropped from analysis. There were 170 EARLI cord blood samples, and 127 EARLI placenta samples with 455,650 probes that passed DNA methylation quality control.

In MARBLES, placenta and cord blood samples were run on the EPIC array at different times and preprocessed separately. First, we dropped cord blood samples from multiple births (cord blood $n=8$ samples). Samples that had mismatched predicted sex were dropped (cord $n=3$). For siblings not from multiple births, all but one sibling was dropped (cord $n=13$). Probes were dropped if they had detection- p ($p>0.01$) failure in greater than 5% of samples ($n=4630$). Cross-reactive probes ($n=42,967$) and Y chromosome probes were dropped from analysis ($n=379$) [60]. There were 243 MARBLES cord blood samples with 817,883 probes that passed DNA methylation quality control. Second, no placenta samples had mismatched predicted sex. There were no samples from multiple births, and all but one sample from siblings were dropped (placenta $n=2$). Probes that failed detection- p in $>5\%$ of samples ($n=1,699$), cross-reactive probes ($n=43,068$), and remaining Y-chromosome probes were dropped from analysis ($n=84$). There were 90 MARBLES placenta samples with 821,008 probes that passed quality control. Sample exclusion is summarized in Additional file 1: Figure S1 and CpG probe exclusion is summarized in Additional file 1: Figure S2.

In cord blood samples, cell type (CD8⁺ T-cell, CD4⁺ T-cell, natural killer cell, B-cell, monocyte, granulocyte, and nucleated red blood cell) proportions were estimated using a combined reference panel with the IDOL method [61]. In placenta samples, EpiDISH [62] was used to predict proportions of placenta cell types using a reference panel from the planet package: trophoblasts, stromal cells, Hofbauer cells, endothelial cells, nucleated red blood cells, and syncytiotrophoblasts [63]. Mean

DNA methylation per person was calculated as the mean across all probes [19]. Mean DNA methylation restricted to probes in genomic regions (CpG island, shore, shelf, or open sea) were also computed. We used Illumina's annotation of CpG sites to assign genomic regions (CpG island, CpG shore, CpG shelf, open sea) [64, 65].

Genetics data processing

In EARLI, genetic data were measured using the Omni5 + exome array (Illumina) at the John Hopkins University Center of Inherited Disease Research (CIDR). Data on 4.6 million single nucleotide polymorphisms (SNPs) were generated for 841 EARLI family biosamples (including maternal, paternal, proband, and infant samples) from 254 families and 18 HapMap control samples. Samples were processed together, but only data from infants with cord blood or placenta methylation were used. No samples had missing genotypes at $>3\%$ of probes, or excess heterozygosity or homozygosity [4 standard deviations]. Probes were removed if they had technical problems flagged by CIDR or missing genomic location information. Single nucleotide polymorphisms (SNPs) with minor allele frequencies $>5\%$ were removed if they had a missingness rate $>5\%$, and SNPs with minor allele frequency $<5\%$ were removed if they had a missingness rate $>1\%$. There were 2.5 million clean SNPs for 827 samples, which were merged with the 1000 genomes project (1000GP, version 5) data [66] and principal components for genetic ancestry were computed.

In MARBLES, SNPs on 643 infant and mother samples from 234 families were genotyped using the Illumina Mega array at the John Hopkins University Center of Inherited Disease Research (CIDR). Maternal and infant samples were processed together, but only data from infants with cord blood or placenta methylation measures were used. We again applied stringent quality control criteria [67] to the raw 1.75 million genotypes to remove low quality SNPs and samples. Our criteria include removal of samples with call rate $<98\%$, sex discrepancy, and relatedness ($\pi\text{-hat}<0.18$) to non-familial samples. We also filtered SNPs with call rates $<95\%$, excess hetero- or homozygosity, and minor allele frequency (MAF) $<5\%$. After quality control, 620 samples and 758 thousand SNPs remained. Principal components were calculated on genotype data, and these principal components were used to adjust for genetic ancestry in models.

Statistical analyses

Study sample descriptive statistics were calculated for each of the four cohort/tissue groups. For continuous covariates (maternal age at delivery, gestational age, estimated cell proportions), we calculated mean and

standard deviation. For categorical covariates (maternal education, infant sex, infant race/ethnicity), we provided number and frequency. We tested for differences in covariates and prenatal vitamin status using t-tests for continuous covariates, and Chi-square tests for categorical covariates. Because of systematic differences in measures, in terms of platform and timing/batches, we chose not to pool the EARLI and MARBLES samples and instead to conduct analyses separately within each sample. We conducted principal component analysis on the DNA methylation data and evaluated principal component associations with covariates. These associations were visualized with a heatmap of p -values.

In multivariable linear regression analyses, first, we examined array-wide mean DNA methylation differences by prenatal vitamin intake in the first month of pregnancy. Regression models were adjusted for infant sex, maternal age, gestational age, maternal education, and genetic ancestry principal components. To provide flexibility in conceptualization of cell type proportions we used estimated cell proportions as terms in regression models. Since cell composition estimates sum up to 100%, to avoid collinearity issues in models, we did not use all predicted cell types in models. For placenta, syncytiotrophoblast and Hofbauer proportions were used, while in cord blood granulocyte and nucleated red blood cell proportions were used. Batch was also adjusted for as a covariate. In the EARLI cohort, samples were measured in two runs, and an indicator variable for run was used for adjustment. MARBLES cord blood was run in a single batch, and sample plate (of which there were three) was used as a covariate for adjustment. MARBLES placenta was run in a single batch on a single plate, and no batch covariate was used. Since smoking has known impacts on DNA methylation, and smoking rates in both cohorts are very low, we excluded mothers with smoking during pregnancy in this analysis (number dropped: MARBLES cord $n=10$, MARBLES placenta $n=4$, EARLI cord $n=6$, EARLI placenta $n=6$). We visualized regression coefficients and 95% confidence intervals using forest plots.

Next, we performed epigenome-wide association analyses by examining single CpG site differential DNA methylation. We fit parallel linear models for each probe. Models were again adjusted for infant sex, maternal age, gestational age, maternal education, genetic ancestry PCs, and estimated cell proportions. Regression and empirical Bayes standard error moderation were performed using the *limma* package [68]. We visualized findings using volcano plots of effect estimates and $-\log_{10}$ (p -values). For sites reaching a nominal p -value threshold ($p < 0.05$), we calculated the proportion of sites that had higher DNA methylation with prenatal vitamin intake and the proportion of sites with lower DNA methylation.

To compare pairwise results across cohort and tissues, we examined Pearson correlations of effect estimates from these regression models, across all sites in common between the 450k and EPIC methylation arrays. We also focused on CpG sites that had p -value < 0.01 in multiple cohort/tissues, examining the overlap of such sites with an upset plot and the Pearson correlation of overlapping sites. For sites prioritized in multiple cohorts/tissues, we also used scatter plots to visualize the effect estimates.

We tested enrichment for gene ontology biological processes using the *missMethyl* package [69]. As input to *missMethyl*, CpG sites with p -value < 0.01 in the epigenome-wide regression models were used. We ranked the gene ontologies by significance, then computed a rank sum by adding the ranks across the four cohort/tissue groups. In addition, we tested for enrichment of chromatin state types using *eFORGE 2.0* [70]. The top 1000 CpG sites for each cohort/tissue analysis was input into the *eFORGE* site, with appropriate array platform chosen (450k for EARLI, EPIC for MARBLES), and Consolidated Roadmap Epigenomics—All 15-state marks and 1 kb window proximity options, with other options set at defaults.

Replication testing

In EARLI, whole genome bisulfite sequencing (WGBS) data were available on 63 cord blood samples sequenced on the HiSeq X [51 overlapping with array methylation data]. Sample processing and WGBS quality control and alignment for cord blood samples [71] and placenta samples [72, 73] have been previously discussed. In MARBLES, WGBS data were available for 91 placenta samples sequenced on HiSeq X Ten (89 overlapping with the array methylation data), 45 cord blood samples sequenced on HiSeq 4000 [30 overlapping with array methylation data], and 42 cord blood samples sequenced on the HiSeq X [35 overlapping with array methylation data].

Raw sequencing reads were preprocessed, mapped to human genome, and converted to CpG methylation count matrices with *CpG_Me* using default parameters [74–76]. Reads were trimmed for adapters and methylation bias, aligned to the reference genome, and filtered for PCR duplicates. Methylation counts at all sites were extracted to *Bismark* cytosine methylation reports. The *CpG_Me* workflow incorporates *Trim Galore*, *Bismark*, *Bowtie2*, *SAMtools*, and *MultiQC* [75, 77–80].

Differentially methylated regions (DMRs) were identified between prenatal vitamin intake during the first month of pregnancy with adjustment for sex and 10 permutation tests using *DMRICH* [74]. The DMR analysis utilizes a smoothing and weighting algorithm to weight regions with high coverage and low variation. Permutation testing was performed on pooled null distribution

to generate empirical p -values as significant DMRs. The DMRichR pipeline utilized dmrseq and bsseq algorithms [81, 82]. To evaluate consistency with array results, we identified array probes within 5 kb of the DMR and examined concordance in estimated directions of effect of those CpG probes and the DMR.

Abbreviations

EARLI: Early Autism Risk Longitudinal Investigation; DHA: Docosahexaenoic acid; DMRs: Differentially methylated regions; LINE-1: Long Interspersed Nuclear Element-1; MARBLES: Markers of Autism Risk Learning Early Signs.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13072-022-00460-9>.

Additional file 1. Prenatal vitamin intake in first month of pregnancy and DNA methylation in cord blood and placenta. This supplementary material contains 7 supplementary figures and 8 supplementary table. An additional 10 large supplementary tables are provided as comma separated value tables.

Additional file 2. Supplemental Table 2. Single site DNA methylation array adjusted regression results: Use of prenatal vitamins in the first month of pregnancy in placenta of EARLI cohort.

Additional file 3. Supplemental Table 3. Single site DNA methylation array adjusted regression results: Use of prenatal vitamins in the first month of pregnancy in placenta of MARBLES cohort.

Additional file 4. Supplemental Table 4. Single site DNA methylation array adjusted regression results: Use of prenatal vitamins in the first month of pregnancy in cord blood of EARLI cohort.

Additional file 5. Supplemental Table 5. Single site DNA methylation array adjusted regression results: Use of prenatal vitamins in the first month of pregnancy in cord blood of MARBLES cohort.

Additional file 6. Supplemental Table 6. Gene ontology ranking by p -value for EARLI placenta, EARLI cord blood, MARBLES placenta, and MARBLES cord blood, ordered by rank sum across the four cohort/tissue groups.

Additional file 7. Supplemental Table 7. Gene ontology enrichment results for MARBLES cord blood.

Additional file 8. Supplemental Table 8. Gene ontology enrichment results for EARLI cord blood.

Additional file 9. Supplemental Table 9. Gene ontology enrichment results for MARBLES placenta.

Additional file 10. Supplemental Table 10. Gene ontology enrichment results for EARLI placenta.

Additional file 11. Supplemental Table 11. Differentially methylated region analysis for whole genome bisulfite sequencing data.

editing. RS provided funding and project conceptualization and project management, manuscript review and editing. KB conceptualized the manuscript and contributed to the original draft and revisions. All authors read and approved the final manuscript.

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Availability of data and materials

Data are available through the National Database for Autism Research (NDAR) accession numbers for EARLI (1600) and MARBLES (2462). Code files are available through GitHub (<https://github.com/bakulskilab>).

Declarations

Ethics approval and consent to participate

The EARLI study was reviewed and approved by Human Subjects Institutional Review Boards (IRBs) from each of the four study sites (Johns Hopkins University, Drexel University, University of California Davis, and Kaiser Permanente Northern California). The MARBLES protocol was reviewed and approved by the Human Subjects IRB from University of California Davis. The University of Michigan Institutional Review Board (HUM00116291) approved these secondary data analyses.

Consent for publication

All co-authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

JD compiled and analyzed the data from the EARLI and MARBLES cohorts and was a major contributor in writing the manuscript. LM was a major contributor in writing the manuscript. YZ generated and analyzed the placenta whole genome bisulfite sequencing data. KB analyzed the genetics data. JF generated and curated data. LC developed the cohort and provided manuscript review and editing. IH-P developed the cohort and provided manuscript review and editing. CN developed the cohort and provided manuscript review and editing. JL provided supervision, and provided manuscript review and editing. DF developed the cohort and provided manuscript review and

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