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Original article

Maternal non-compliance with recommended folic acid supplement use alters global DNA methylation in cord blood of newborns: A cohort study

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SUMMARY

Background & aims: Prenatal folate exposure may alter epigenetic marks in the offspring. We aimed to evaluate associations between prenatal exposure to folic acid (FA) in preconception and in utero with cord blood DNA methylation in long interspersed nuclear element 1 (LINE-1) and Alu short interspersed nuclear elements (SINEs) as markers of global DNA methylation levels.

Methods: Data come from 325 mother–child pairs participating in the Nutrition in Early Life and Asthma (NELA) birth cohort (2015–2018). Pregnant women were asked about supplement use, including brand name and dose, one month before pregnancy (preconception) and through the trimesters of pregnancy. Maternal dietary folate intake was assessed using a validated food frequency questionnaire with additional questions for FA supplement use. Folate serum levels were measured in mothers at 24 weeks of gestation and in cord blood of newborns. DNA methylation was quantitatively assessed by bisulfite pyrosequencing on 5 LINE-1 and 3 Alu different elements. Associations were estimated using multivariable linear regression models.

Results: A reduction in methylation levels of LINE-1 in newborns was associated with the use of FA supplements below the recommended doses (<400 ug/day) during preconception (−0.50; 95% CI: −0.91, −0.09; P = 0.016), and from preconception up to 12 weeks of gestation (−0.48; 95% CI: −0.88, −0.08; P = 0.018). Maternal use of FA supplements above the tolerable upper intake level of 1000 ug/day from preconception until 12 weeks of gestation was also related to lower methylation in LINE-1 at birth (−0.77; 95% CI: −1.52, −0.02; P = 0.044). Neither FA supplement use after 12 weeks of gestation nor maternal total folate intake (diet plus supplements) were associated with global DNA methylation levels at birth.

Abbreviations: FA, folic acid; FFQ, Food Frequency Questionnaire; LINE, long interspersed nuclear element; NELA, Nutrition in Early Life and Asthma.

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Conclusions: Maternal non-compliance with the use of FA supplement recommendations from preconception up to 12 weeks of gestation reduces offspring global DNA methylation levels at birth.

1. Introduction

Growing evidence indicates that changes in epigenetic marks before birth may play a key role in human development and in the occurrence of diseases later in life. In this sense, the interest on the role of transposable elements, repetitive genomic elements that harbor binding sites for human transcription factors, has increased recently [1,2]. DNA sequences of these repetitive elements account for 50% of the human genome and have often been studied as surrogates of global DNA methylation status [3].

Among retrotransposons, the long interspersed nuclear elements (LINEs) and the short interspersed nuclear elements (SINEs) Alu represent the two most abundant human repetitive elements sequences - they constitute at least one-third of the human genome - and the most studies families [4–6]. The mobility of these elements around the genome generates both intra- and inter-individual genetic variation in the human population and can also result in abnormal disfunction and disease if insertions occur into important conserved sequences [7]. Interestingly, high level of methylation, typically observed in LINE-1, inhibits transposition [8] and constitutes an important mechanism of regulation and defense against this phenomenon specially during critical periods of fetal development when insertions of transposable elements seem to be particularly active [5,8]. Conversely, demethylation can activate retrotransposition of these genomic elements [7].

Maternal nutrition is a major determinant of fetal development and growth. An adequate supply of different micronutrients such as folic acid (FA) or folate (natural form) is crucial during pregnancy to assure maternal health and offspring growth and development as well as health later in life [9]. Currently, in addition to an appropriate dietary folate intake, a periconceptional use of FA supplements of 400 ug/day (i.e., from at least one month before conception out to 12 weeks of pregnancy) is recommended worldwide to reduce the risk of neural tube defects (NTDs) [10]. Moreover, growing evidence indicates that maternal FA supplementation could also reduce the risk of other congenital malformations [11] and adverse health outcomes in the offspring, including neurodevelopment [12,13].

Mechanisms involved in the association between prenatal exposure to FA and health outcomes later in life are not well understood, but epigenetic changes have emerged as potential molecular mechanisms to explain the beneficial effects of FA to prevent NTDs [14,15] and the associations between maternal FA status and other health outcomes at birth and later in life [16]. In this sense, animal studies have reported that FA deficiency during fetal development impairs one-carbon metabolism, leading to LINE-1 hypomethylation in fetal cells and tissues [17,18]. However, currently available evidence in humans regarding the impact of prenatal exposure to FA supplements on global DNA methylation status in the offspring remains limited [19,20]. A birth cohort study did not find evidence that FA supplement use in the periconceptional period, and up to 12 weeks of gestation, influenced LINE-1 status in cord blood of the offspring; however, supplement use after 12 weeks was associated with reduced methylation in LINE-1 [19]. A randomized controlled trial found that continued FA supplementation (400 μg/d) through the second and third trimesters resulted into lower overall DNA methylation levels at LINE-1 in cord blood of newborns compared to placebo [20].

In this prospective study, we examined whether prenatal exposure to FA from preconception and along pregnancy is associated with changes in methylation levels of LINE-1 and Alu in cord blood of newborns as markers of global DNA methylation status at birth.

2. Materials and methods

2.1. Study population

Methods of the NELA ("Nutrition in Early Life and Asthma") prospective birth cohort study have been previously described in detail [21]. In brief, recruitment of participants took place between 2015 and 2018 during the routine 20th week echography control at the "Virgen de la Arrixaca" University Clinical Hospital (Murcia, Spain), where 738 pregnant women were recruited. Inclusion criteria were: Caucasian and Spanish origin; 18–45 years of age; living in Health Area I (suburban and rural) or in certain districts of Health Areas VI and VII (mainly urban) of the Region of Murcia; planning to live in the area of study during at least 2 years; singleton pregnancy; spontaneous conception; intention to deliver in the aforementioned hospital; and normal ultrasound findings at the time of the visit (no major fetal malformations). Exclusion criteria included: chronic disease in the mother, such as pregestational diabetes mellitus or other major endocrine disorders, pregestational hypertension, autoimmune disease, or cancer; and verbal communication problems. Among them, markers of global DNA methylation in cord blood were assessed in 373 newborns (50.5%) of which 48 did not have information on maternal FA supplements use. Thus, the current study is based on 325 mother–child pairs with complete information (Supplementary Fig. 1). The study protocol was reviewed and approved by the Ethics Committee of the Biomedical Research Institute of Murcia (IMIB-Arrixaca) in accordance with the guidelines of The Declaration of Helsinki (report 9/14; September 29, 2014). Written informed consents were obtained from parents at recruitment.

2.2. Assessment of prenatal exposure to folic acid

Maternal use of FA supplements was retrospectively assessed through questionnaires administered during personal interviews at two time points of gestation: at 20–24 weeks and at 32–35 weeks. Information on the use of supplements during the month before pregnancy was obtained the question "Did you use supplements of folic acid during the previous month to conception?" Information on the use of supplements during each trimester of pregnancy was collected throughout the following two questions: 1) "Have you used supplements of folic acid during the first, second and third trimester of pregnancy?"; and 2) "Have you used iron supplements during the first, second and third trimester of pregnancy?". For all those questions, if a positive answer was obtained, we asked the participants to choose the supplements they used before or during pregnancy from a list of brands of supplements that were commonly available in Spain and to further tell the brand and the

frequency of use and kind of supplement they consumed, if it was not shown in the list. Subsequently, FA supplements use was estimated based on supplement brand name and composition, frequency of use (0.5 times/day; 1 time/day; 2 times/day), and timing of consumption (previous month to conception and trimesters of pregnancy). Maternal FA supplements use was calculated for different time windows including preconception (previous month), from preconception until 12 weeks of gestation, and during the first, the second and the third trimester of pregnancy, and categorized as <400 ug/day, 400–999 ug/day (this was used as the reference category in the analyses) and ≥1000 ug/day.

We also estimated maternal dietary intake folate intake during pregnancy. For this purpose, a validated food frequency questionnaire (FFQ) [22] was administered to participants at 20–24 weeks of gestation. According to current recommendations maternal total FA intake (dietary folate plus FA from supplements) from preconception period up to 20 weeks of gestation was calculated and categorized into two categories: <600 ug/day and ≥600 ug/day [23]. FA food fortification was not voluntary in Spain during the study, thus, the differences in bioavailability of added FA and naturally present folate in foods were not taken into account in the calculation of total folate intake of the participants.

In addition, serum levels of folate were measured in maternal fasting blood collected at 24 weeks of gestation and in cord blood of newborns collected at birth. Serum folate concentrations were analyzed by using the competitive electrochemiluminescence immunoassay on the ADVIA Centaur Immunoassay System (SIEMENS). According to current evidence on serum levels of folate in women of childbearing age to prevent fetal malformations, concentrations were categorized into two categories: ≤11.22 ng/ml vs. >11.22 ng/ml [24].

2.3. Measurement of global DNA methylation levels in cord blood of newborns

Umbilical venous cord blood samples were collected at birth and stored at −80 °C until processing. DNA was extracted from cord blood buffy coat using the Maxwell DNA Blood SEV kit with the automated extraction equipment Maxwell 3000, according to the manufacturer's protocol. DNA methylation was quantified by bisulfite-PCR and pyrosequencing, according to the methods previously developed [25,26], in the Genomics Core Facility of the Biomedical Research Institute of Murcia (IMIB). DNA (500 ng) was converted with bisulfite using the EZ DNA Methylation kit (Zymo Research), following the manufacturer's protocol. PCR amplification of DNA methylation of LINE-1 and Alu elements was performed in a PyroMark Q24 System (Qiagen) according to designs previously described [27]. Specific details of the pyrosequencing methods such as the PCR primers and conditions, sequencing primers and nucleotide dispensation are shown in Supplementary Table 1. PCR amplification of LINE-1 and Alu elements was performed using 25 ng of bisulfite converted DNA, 2 μM of each primer and the PyroMark PCR Master mix of the PyroMark PCR kit (Qiagen), in a final volume of 25 μL. In both methods, the size of the resulting PCR products was approximately 150 bp, as assessed by electrophoresis of 2 μL of each PCR reaction on 2 % agarose gels. A no-template control was included in each PCR plate and checked for negativity on agarose gels. Each PCR reaction was split into two pyrosequencing reactions. Thus, the PCR products (2 × 10 μL) were bound to Streptavidin Sepharose High Performance (GE Healthcare), then denatured and purified using PyroMark buffers (Binding, Annealing, Denaturation, and Wash) in a PyroMark Q24 MDx Vacuum Workstation. Finally, sequencing was performed using PyroMark Gold Q24 Reagents in a PyroMark Q24 MDx instrument. The pyrosequencing runs were analyzed using the PyroMark Q24 software. The second nucleotide dispensed in the LINE-1 runs (dCTP, Supplementary Table 1) represents an internal control for bisulfite treatment. Human Methylated & Non-Methylated DNA Set (Zymo Research) were also included in each PCR and pyrosequencing plate as controls, positive and negative, respectively, for the pyrosequencing assays. In addition, all plates contained randomly coded samples from different age groups to reduce the risk of plate bias. Methylation of each CpG site was calculated twice. Coefficient of variation (CV) for duplicate measurements was calculated and if the average was >5 % the plate was repeated. Global DNA methylation variables were the result to average duplicate measurements on different CpG sites. Firstly, we obtained a mean value of each CpG site (mean of duplicated measurements) and then, the mean values of 3 CpGs for LINE-1 and of 5 CpGs for Alu (Supplementary Table 2). Intra-class correlation coefficients between duplicated values of LINE-1 and Alu methylation were 0.81 (95%CI = 0.76; 0.85) and 0.90 (95%CI = 0.87; 0.92), respectively.

2.4. Other variables

The following variables were considered a priori potential confounders because of their possible association with global DNA methylation levels and prenatal exposure to folate: maternal age, maternal educational level (incomplete secondary or less, complete secondary and university), maternal social class (occupation during pregnancy using a widely used Spanish adaptation of the international ISCO88 coding system) [28] (I-II, managers/technicians; III, skilled; IV-V, semi-skilled and non-skilled; and unemployed), maternal pre-pregnancy body mass index based on measured height at recruitment and pre-pregnancy self-reported weight (kilograms per square meter, kg/m2) (normal weight <25, overweight 25–29.99, and obese ≥30), area of residence (urban, residential and rural), maternal smoking and alcohol use during pregnancy (no vs. yes), self-reported physical activity during pregnancy (sedentary, poorly active, and moderately-strongly active), parity (0 vs. 1 or more), previous gestations (0 vs. 1 or more), type of delivery (vaginal versus cesarean section), child sex, gestation age at birth (weeks), and birth weight. Information was obtained from questionnaires administered face-to-face to participants by trained interviewers or retrieved from clinical records.

2.5. Statistical analysis

For comparisons between continuous variables Student's t-test or Mann–Whitney's U tests were used to analyze normally and non-normally distributed quantitative variables, respectively. In contingency tables, Chi-2 test was applied to assess the relationship between two categorical variables. Correlations between serum folate concentrations and maternal FA supplement usage were assessed by Spearman rank correlation coefficients. Multivariate linear regression models were used to examine the association between prenatal FA exposure and mean levels of DNA methylation at LINE-1 and Alu repetitive elements in cord blood of newborns. Final models were adjusted for newborn sex, birth weight, maternal age, maternal education level, maternal social class, and maternal smoking in pregnancy. Models were further adjusted for estimated cell proportions of CD8T, CD4T, NK cells, B cells, monocytes, granulocytes, and nuclear red blood cells [29] as a sensitivity analysis to minimize potential effect of cellular composition differences in immune cells on measured global DNA markers. Analyses were performed using Stata Software (version 15.1, StataCorp, College Station, Texas).

3. Results

Main characteristics of study participants are shown in Table 1. Compared with excluded participants, mothers included in the present study were older and had higher social class. Included newborns had longer gestational age and higher birth weight than those excluded. No differences were observed in other main characteristics.

Twenty-eight percent of pregnant women used a folate dose from supplements of 400–999 ug/day and 68.6% used doses of FA supplements below the recommendations (<400 ug/day) during the preconception period (Table 2). Similarly, 30% of pregnant women used a folate dose of 400–999 ug/day from supplements and 63% used unsatisfactory FA doses from supplements from preconception up to 12 weeks of gestation. Throughout pregnancy, the percentage of women using a folate dose of 400–999 ug/day from supplements increased to 86% during the first and second trimesters and to 83% during the third trimester (Table 2). Forty-five percent of pregnant women had an adequate total intake (diet plus supplements) of FA from preconception until 20 weeks of gestation. Maternal FA supplement usage and dietary intake weakly correlated with serum folate concentrations of mothers at mid-pregnancy (range: 0.047–0.199) and of newborns' cord blood (range: 0.074–0.237) (Supplementary Table 3). Correlation between maternal and cord blood serum folate concentrations was also weakly positive (coefficient 0.308) (Supplementary Table 3).

Average DNA methylation levels for the repetitive elements analyzed are presented in Supplementary Table 2. DNA methylation levels in CpG positions of LINE-1 ranged from 71% to 84%, showing higher levels among males compared to females. DNA methylation levels in CpG positions of Alu ranged from 13% to 32%, with no differences between females and males.

Crude associations between prenatal expose to FA and DNA methylation levels at birth in global markers are shown in Supplementary Table 4. After adjusting for potential confounders crude associations did not materially change (Table 3). We found a reduction in methylation levels of LINE-1 in newborns to be associated with insufficient FA supplement use (<400 ug/day) during preconception (−0.50; 95%CI: −0.91, −0.09; P = 0.016) and up to 12 weeks of gestation (−0.48; 95% CI: −0.88, −0.08; P = 0.018). Moreover, maternal FA supplement use above the recommended dose (≥1000 ug/day) from preconception until 12 weeks of gestation was related to lower methylation levels in LINE-1 in newborns (−0.77; 95%CI: −1.52, −0.02; P = 0.044). Neither FA supplement

Table 1
Main characteristics of included and excluded study participants. The NELA cohort study (2015–2018).

Maternal characteristics	Included (n = 325)	Non-included (n = 413)	P value
Age (years)	33.1 ± 4.2	32.2 ± 4.9	0.007
18-25	16 (4.9)	41 (9.9)	0.040
26-35	221 (68.0)	269 (65.1)	
36+	88 (27.1)	103 (24.9)	
Education level			
Incomplete secondary or less	56 (17.2)	90 (21.7)	0.090
Complete secondary	78 (24.0)	113 (27.4)	
University	191 (58.8)	210 (50.9)	
Social class			
I-II, managers/technicians	133 (40.9)	131 (31.7)	0.009
III, skilled	79 (24.3)	88 (21.3)	
IV-V, semi-skilled and non-skilled	53 (16.3)	91 (22.0)	
Unemployed	60 (18.5)	103 (24.9)	
Pre-pregnancy BMI (kg/m^2)	24.2 ± 4.5	23.7 ± 4.4	0.104
Normal (<25)	216 (66.7)	293 (72.0)	0.281
Overweight (25–29.99)	79 (24.4)	81 (19.9)	
Obesity (≥30)	29 (8.9)	33 (8.1)	
Area of residence			
Urban	232 (71.4)	302 (73.1)	0.040
Residential	40 (12.3)	67 (16.2)	
Rural	53 (16.3)	44 (10.7)	
Tobacco Smoker	55 (16.9)	73 (17.7)	0.789
Alcohol consumption, yes	21 (6.5)	22 (5.3)	0.514
Physical activity			
Sedentary	60 (18.5)	59 (14.3)	0.231
Poorly active	134 (41.2)	190 (46.0)	
Moderately-strongly active	131 (40.3)	164 (39.7)	
Parity, nulliparous	161 (49.5)	213 (51.6)	0.583
Previous gestations			
0	127 (39.1)	179 (43.3)	0.243
+1	198 (60.9)	234 (56.7)	
Type of delivery			
Vaginal	261 (80.3)	294 (76.0)	0.164
Cesarean section	64 (19.7)	93 (24.0)	
Newborns characteristics			
Sex			
Male	171 (52.6)	186 (47.1)	0.140
Female	154 (47.4)	209 (52.9)	
Gestational age (wks)	39.8 ± 1.3	39.4 ± 1.7	0.002
Birthweight (g)	3298.6 ± 427.4	3194.6 ± 506.8	0.003
<2500	12 (3.7)	30 (7.8)	0.065
2500-3999	298 (91.7)	337 (87.1)	
≥4000	15 (4.6)	20 (5.2)	

Values expressed as means ± SDs or n (%). Differences between groups were assessed using Student's *t*-test (continuous variables) or Chi-2 test (categorical variables). Newborn sex n = 720 and birth weight n = 712 due to missing values.

Table 2
Descriptive of prenatal exposure to folic acid (FA). The NELA cohort study (2015–2018).

		Prenatal exposure to folic acid		
Maternal use of FA from supplements	**N**	**<400 ug/day**	**400–999 ug/day**	**≥ 1000 ug/day**
Preconception (previous month)	325	223 (68.6)	91 (28.0)	11 (3.4)
From preconception until 12 weeks	325	205 (63.1)	98 (30.2)	22 (6.8)
First trimester of pregnancy	325	24 (7.4)	280 (86.1)	21 (6.5)
Second trimester of pregnancy	325	26 (8.0)	282 (86.8)	17 (5.2)
Third trimester of pregnancy	321	39 (12.1)	268 (83.5)	14 (4.4)
Maternal total folate and FA intake (diet plus supplements)		**< 600 ug/day**	**≥ 600 ug/day**	
From preconception until 20 weeks	325	177 (54.5)	148 (45.5)	
Serum folate concentration		**≤11.22 ng/ml**	**>11.22 ng/ml**	
Mothers at mid-pregnancy (24 weeks)	323	103 (31.9)	220 (68.1)	
Newborns cord blood at birth	291	5 (1.7)	286 (98.3)	

Table 3
Associations[a] between prenatal exposure to folic acid (FA) and global DNA methylation levels in cord blood (n = 325). The NELA cohort study.

	LINE-1		Alu	
	Coeff. (95% CI)	p value	Coeff. (95% CI)	p value
Maternal FA supplement use (μg/day) during preconception				
400-999	Ref.		Ref.	
<400	−0.50 (−0.91, −0.09)	0.016	0.06 (−0.09, 0.22)	0.426
≥1000	−0.53 (−1.54, 0.49)	0.307	0.03 (−0.36, 0.42)	0.889
Maternal FA supplement use (μg/day) from preconception until 12 weeks				
400-999	Ref.		Ref.	
<400	−0.48 (−0.88, −0.08)	0.018	0.11 (−0.04, 0.27)	0.140
≥1000	−0.77 (−1.52, −0.02)	0.044	0.16 (−0.13, 0.45)	0.284
Maternal FA supplement use (μg/day) first trimester				
400-999	Ref.		Ref.	
<400	−0.35 (−1.03, 0.33)	0.308	0.05 (−0.21, 0.31)	0.683
≥1000	−0.67 (−1.39, 0.05)	0.069	0.05 (−0.22, 0.33)	0.709
Maternal FA supplement use (μg/day) second trimester				
400-999	Ref.		Ref.	
<400	−0.30 (−0.95, 0.36)	0.371	0.05 (−0.20, 0.30)	0.689
≥1000	−0.46 (−1.26, 0.35)	0.265	0.08 (−0.22, 0.39)	0.587
Maternal FA supplement use (μg/day) third trimester				
400-999	Ref.		Ref.	
<400	−0.52 (−1.07, 0.03)	0.064	0.07 (−0.14, 0.28)	0.505
≥1000	−0.29 (−1.17, 0.59)	0.520	0.12 (−0.21, 0.46)	0.474
Maternal total FA intake (μg/day) from preconception until 20 weeks				
≥600	Ref.		Ref.	
<600	0.06 (−0.30, 0.41)	0.760	0.005 (−0.13, 0.14)	0.944
Maternal serum folate (ng/ml)				
>11.22	Ref.		Ref.	
≤11.22	−0.18 (−0.57, 0.22)	0.374	−0.01 (−0.16, 0.14)	0.912
Newborn cord blood folate (ng/ml)				
>11.22	Ref.		Ref.	
≤11.22	1.05 (−0.41, 2.51)	0.157	−0.09 (−0.63, 0.46)	0.750

[a] Models adjusted for newborn sex, birth weight, maternal age, maternal education level, maternal social class, and maternal smoking in pregnancy.

use after 12 weeks of gestation nor maternal total intake (diet plus supplements <600 ug/day) of FA from preconception until 20 weeks of gestation were associated with global DNA methylation levels at birth.

Maternal and newborn serum folate levels were not related to cord blood global DNA methylation levels in LINE-1. In addition, no associations were found between maternal supplement use of FA nor maternal and newborn circulating levels of folate with methylation levels in Alu (Table 3).

Further adjustment for estimated immune cell proportions in cord blood did not materially change the estimations (Supplementary Table 5).

4. Discussion

In this prospective birth cohort study, we found that a maternal FA supplement use lower than 400 ug/day from preconception up to 12 weeks of gestation was associated with reduced LINE-1 methylation levels in cord blood of newborns. Moreover, maternal FA supplement use above the tolerable upper limit of 1000 ug/day from preconception until 12 weeks of gestation was also related to lower methylation levels in LINE-1 at birth. Neither maternal FA supplement use in other prenatal temporal windows (i.e., after 12 weeks of gestation) nor recommended maternal total intake of FA (i.e, diet plus supplements) were related to changes in LINE-1 methylation in cord blood of newborns. In addition, Alu methylation levels in cord blood of newborns were not associated with maternal FA use.

Our study detected that the percentage of women who used lower than recommended doses (400 μg/day) of FA supplements ranged from 68% (during preconception) to 63% (from preconception to 12 weeks of gestation), which is in accordance with results from previous studies carried out in developed settings. For example, a previous study carried out among Spanish pregnant women from four different geographical areas showed that almost 60% of women did not take the recommended dose of FA from supplements during the periconception period [30]. Similar figures have been reported in studies carried out in other European

countries, including Poland [31] and the UK [32] showing that 42.8 and 55% of mothers did not use FA supplements in the periconceptional period.

We found that boys had significantly higher levels of methylation in all the LINE-1 elements compared to girls, similarly to previous studies conducted in cord blood of newborn and in total blood in childhood [33,34], with methylation differences in the same range of what we have found (around 1% increase). This result is also consistent with those observed in adults, finding lower methylation in women than men [35,36]. These differences are likely due to the relationship of LINE-1 with X chromosome inactivation. It has been found that individual LINE-1 loci located on the X chromosome as well as some in autosomes show differential methylation in males and females primarily located in the X chromosome [37]. It has also been postulated that hypomethylation could be induced in the chromosome X or less resources are available to maintain a whole chromosome inactive among females to properly methylate autosomal loci [38].

To our knowledge only two previous studies have investigated the relationship between maternal FA supplement use and changes in global DNA methylation levels in the offspring at birth [19,20]. Although both studies reported that continued FA supplement use of 400 μg/d after 12 weeks of gestation resulted into reduced DNA methylation levels at LINE-1 in cord blood of newborns [20], we did not find significant differences during this time windows of pregnancy; however, the direction of the associations in our study was the same. There are several potential explanations for the lack of association of maternal FA supplement use during specific trimesters of pregnancy with global DNA methylation levels in our study. Methodological differences in the assessment of DNA methylation (i.e. number of CpG sites analyzed) could hinder comparisons between studies. Moreover, there are few pregnant women in our population study who did not accomplish the recommended supplement FA doses after conception. For example, only 24 (7.4%), 26 (8.0%) and 39 (12.1%) of women had dosages of less than 400 ug/day of FA supplements in the first, second and third trimester of pregnancy, respectively. This could result into a lack of power to detect true effects if they really exist. In addition, current results, if true, identify the time window from the preconception period up to 12 weeks of gestation as the most susceptible to maternal FA deficiency effects on global DNA methylation status in the offspring measured as LINE-1 levels. In this sense, periconceptional FA intake is associated with the establishment of DNA methylation in offspring, and early pregnancy is considered as a sensitive period of plasticity in fetal developmental programming and has thus been of interest for several epigenetic studies of maternal diet and offspring DNA methylation in specific genes [39–41].

In addition, our results do not support an association between total (i.e., diet plus supplements) maternal intake of FA and DNA methylation levels in cord blood of newborns. This finding agrees with a previous study that reported maternal dietary folate intake periconceptionally and during the second trimester of pregnancy not to be associated with cord blood LINE-1 methylation [42]. In this sense, it is to be noted that the synthetic oxidized form of folate (vitamin B-9) is considered more bioavailable than dietary folate [43]. It is estimated that food folate bioavailability is 50% and that FA bioavailability is 85% when taken with food (either as fortified food or supplement) or 100% when taken on an empty stomach with water [44]. Thus, folate derived from fortified foods or from supplements is either 1.7 (85/50) or 2.0 (100/50) times, respectively, as bioavailable as naturally occurring food folate. Moreover, distinct relation for each FA source in contributing to the early establishment of DNA methylation has also been reported [45]. In addition, we did not take into account differences in bioavailability of the different folate forms in the calculation of total folate intake of the participants, which might also explain the lack of association between total folate intake and DNA methylation of LINE-1.

Maternal and newborn's serum folate concentrations were weakly correlated with maternal FA supplement usage and dietary intake. Neither maternal nor newborns' cord blood serum concentrations were related to changes in levels of global DNA methylation in newborns. There are several potential explanations for this. Serum folate reflects primarily recent intake [46], whereas estimated intakes from supplements and diet reflect a measure of long-term folate status. In addition, serum concentrations relied on one sampling measurement, which could result into fluctuations in a dietary biomarker concentration with a short half-life.

To our knowledge, this is one of the largest studies in humans investigating the relationship between prenatal expose to FA and global DNA methylation changes in the offspring at birth. Another unique feature was that we examined different time windows of prenatal development including preconception and specific trimesters of pregnancy, and we considered total maternal FA intake (i.e., intake from supplements and diet). Yet, another strength was its population-based, prospective design and the extensive information on potential confounders.

Our study has some limitations. Firstly, the study was conducted in a subsample of the full cohort, which could result into some selection bias. Although some differences were observed in the distributions of several variables between included and excluded participants, these differences are not likely to have affected the validity of the results, because the variables were controlled in the analysis by multivariate adjustment. Secondly, the retrospective assessment of maternal FA supplement use through self-reporting could result into some recall bias as well as misclassification of participants skipping the dose. Thirdly, using an FFQ for dietary assessment likely introduced some measurement error into our estimations; however, this FFQ was validated for pregnant women for the nutrients we examined [22], and thus, it should accurately rank individuals' intake of these nutrients. Fourthly, the magnitude of detected differences in global methylation levels in cord blood of newborns in relation to prenatal exposure to FA supplements was small. However, these differences are in accordance with previous studies showing that transcriptional alterations including at imprinted genes can result from small DNA changes after drug treatment [47]. Finally, we did not examine any adverse health outcome in the offspring nor changes in gene/protein expression or functional changes. In addition, an epigenome-wide DNA methylation analysis is warranted to provide more objective results by identifying DNA methylation changes at specific genes.

5. Conclusion

In conclusion, our results highlight: 1) still a high proportion of childbearing women do not accomplish with the current recommendations on FA supplement use during preconception and pregnancy; and 2) maternal non-compliance with FA supplement use recommendations from preconception up to 12 weeks of gestation may reduce global DNA methylation levels in the offspring. Our findings suggest that changes in global DNA methylation status at birth might mediate adverse effects observed in the offspring because of maternal non-compliance with current recommendation FA supplement use during susceptible time windows of development. In clinical practice, medical staff of developed settings should increase efforts to warrant compliance of recommended FA supplement use among childbearing women before conception and throughout pregnancy to limit potential adverse health outcomes in the offspring programmed via demethylation of transposable elements.

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Author contributions

EM and L G-M conceived and supervised the project and recruited the participants. C M-G, MS-P, CS-M, and JV collected and provided dietary data. JM, EA, and A C-T conceived the experiments and provided the epigenetic data. MT P–S, MJ C and E del C collected the biological samples. EM, MT P–S, and D V-G conceived the study. EM conducted the statistical analyses and wrote the article. All authors have reviewed the results and have critically revised the final version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2024.04.007.

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