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Epigenetic regulation of folate receptor-α (FOLR1) in human placenta of preterm newborns

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ABSTRACT

Introduction: Folates are essential nutrients for fetal development and pregnancy outcomes; they are transported to the fetus during gestation through specific folate transporters located in the placenta. In preterm newborns, we previously showed a lower placental mRNA expression of FOLR1 along with higher folate and lower vitamin B12 cord blood levels. Thereby we aimed to explore *FOLR1* methylation in placentas of preterm newborns and hypothesized an increased *FOLR1* methylation associated with cord blood folates and vitamin B12 concentrations.
Methods: FOLR1 methylation and mRNA were determined by methylation sensitive – high resolution melting (MS-HRM) and by real-time PCR respectively, in two placental sides of placental tissues: maternal (basal, BP) and fetal plates (chorionic, CP) of moderate preterm infants (32-36 gestational age) and term birth (37-41 gestational weeks). Folates and vitamin B12 were determined by electrochemiluminescence in umbilical cord blood samples from term and preterm newborns.
Results: We found that in preterm newborns, FOLR1 mRNA was lower in both plates of placenta compared with term newborns ($p < 0,05$) and was negatively associated with methylation of *FOLR1* in CP. Preterm newborns presented higher folate and lower vitB12 concentrations in cord blood which correlated with increased placental *FOLR1* methylation.
Discussion: In preterm newborns, placental *FOLR1* expression is regulated by epigenetic mechanisms and presumably by maternal concentrations of folate and vitamin B12.

1. Introduction

Folates, including the synthetic form, folic acid (FA), are hydrosoluble molecules of the vitamin B9 family. They are essential for fetal growth and placental development; its deficiency has been related to low birth weight, preterm birth, spontaneous abortion and other altered birth outcomes such as neural tube defects (NTD) [1–5]. Preterm birth is defined as <37-week gestation at birth and constitutes a major public health concern with reported rates ranging from 5% to 20% worldwide [5,6]. Preterm birth is not only a major determinant of neonatal mortality and morbidity but is also associated with long-term adverse health and social consequences [7]. Deficient maternal folate and vitamin B12 intake along with a lower bioavailability of both vitamins reduce their transport to the fetus through the placenta and are included in the etiology of preterm [8,9]. Folates are nutrients naturally present in some foods, and synthetic FA is used both, to fortify food and as nutritional supplement during pregnancy in Chile [10].

Maternal dietary methyl donor intake, and specifically folate and vitamin B12 deficiency predict a poor pregnancy outcome [8,11]. Both vitamins are required for the conversion of homocysteine to methionine, which participates in the synthesis of S-adenosylmethionine (SAM), the main cellular methyl donor for different methylation reactions in the cell [12]. Therefore, it can be concluded that intracellular concentrations of folate and vitamin B12 may be indirectly related to gene methylation, which in turn could affects its expression [13].

Placental transport of folates from mother to the fetus is highly dependent on maternal folate concentrations [14,15]. Three main components participate in this process: the folate receptor α isoform (folate receptor α; FRα/FOLR1); the transporter of reduced folates (reduced folate carrier; RFC) and the folates transport coupled to protons (proton-coupled folate transporter; PCFT/HCPI) [14,16]. The placental expression of these folate transporters is detected in early and

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late pregnancy and may change during the course of pregnancy [16–18]. Each transporter has affinity for a specific chemical form of folates, and in the placenta, the main function of them is to provide enough folate concentrations to ensure sufficient quantities according to fetal development demands.

In a recent study, we have shown that gestational age was positively correlated with FOLR1 mRNA expression, although negatively with protein abundance of FOLR1 in placental tissue [19]. At present, it is unknown whether epigenetic modifications, specifically DNA methylation of the *FOLR1* gene, are involved in the mRNA expression of FOLR1 in placentas of preterm newborns.

FOLR1 is a membrane glycoprotein of 42 kDa with a key role in folates internalization; it is composed by 257 amino acids, and is encoded by the *FOLR1* gene [20]. The *FOLR1* gene is formed of seven exons and 2 independent tissue-specific promoters, promoter 1 (P1) at the exon 1 and promoter 4 (P4) at the exon 4 [21] whose utilization is tissue-specific. More recent studies have found that placental *FOLR1* possess a small and unique CpG island in the codifying region with an inverse relationship between mRNA and methylation towards the extreme 5′-3′related to the CpG island [22].

The hypothesis of the present work states that in placentas of preterm newborns the expression of *FOLR1* is lower than in term placentas, due to changes in the specific methylation of the gene. In addition, we expect that this effect would be associated with folates and vitamin B12 concentrations in their cord blood.

2. Materials and methods

2.1. Experimental design

The Scientific Ethics Committee of the Central Metropolitan Health Service, the Ethics Committee of the Faculty of Medicine and INTA, University of Chile, approved the study. The mothers enrolled in this study were aware of the characteristics and aims of it and voluntarily signed an informed consent.

Pregnant women were recruited during their first control at first trimester at the Hospital San Borja Arriarán in Southern Santiago, Chile. Pregnancies were monitored periodically throughout gestation. We collected samples of 39 placentas of healthy pregnancies randomly selected to meet the inclusion criteria. Placentas were classified according to gestational age as moderate preterm (n = 23; 32-36 gestational age), and term birth (n = 16; 37-41 gestational weeks).

Exclusion criteria included pregnancies <32 gestation weeks, diabetes, hypertension, preeclampsia, smoking (consumption >5 cigarettes/day), alcoholism or drug use, twin or multiple pregnancies and genetic diseases in the newborn.

2.2. Human placental tissues and placental explants

All placentas were collected at delivery; immediately, 10 g explants of chorionic (CP) and basal plates (BP) were extracted from placentas of term and preterm pregnancies. Explants from both sites were rectangular pieces (approximately 7 × 5x0.5 cm) obtained after a cross-section near the umbilical cord as previously reported [17,19,23]. The pieces were immediately washed with fresh cold saline solution to remove excess of blood and were individually frozen in cryotubes under liquid nitrogen and then maintained at −80 °C until analyses.

2.3. DNA isolation and bisulfite treatment

Samples of BP and CP of placentas were pulverized in dry ice. Then, DNA was extracted with a kit DNA easy using columns (Kit DNeasy Blood & Tissue QIAGEN) following manufacturer's instructions. DNA integrity was confirmed by electrophoresis in agarose gels and ethidium bromide, and bands quantification were assessed by spectrophotometry using the Qubit dsDNA BR kit (Termo Fisher Scientific) and the Qubit Fluorometer 2.0 (Invitrogen). Subsequently, 10 μl (50 ng/μl) of DNA samples were treated with sodium bisulfite with the EpiTect Bisulfite kit (QIAGEN) according to the manufacturer's instructions.

2.4. Methylation by MS-HRM (methylation sensitive – high resolution melting)

This method detects methylation variations in the double strand DNA, in PCR amplifications according to their dissociation behavior. It allows discriminating samples according to their CG content by detecting changes in the DNA melting curves visualized with specific staining for DNA. We used the nucleotide sequence GenBank U20391.1 for the *FOLR1* gene. The following primers were designed using the MethPrimer application [24]; forward primer: GGT TAG GAT GGT TTT GAT TTT TTA GTT; reverse primer: ACC CCA AAC TAA ATA CAA TAA CTT ACT T (GenBank: U20391.1). Primers were designed to produce an amplicon of 263 pb corresponding to the *FOLR1* island of CG considering sites 2508, 2518 and 2524 as susceptible for methylation, respect to the site of transcription initiation (Fig. 1). The amplification of PCR was accomplished with 1 μl of bisulfite-treated DNA and 5 μl of KAPA HRM FAST qPCR kit plus 1 μl of 25 mM $MgCl_2$ (Kappa Biosystems). The HRM analysis was performed with the Rotor Gene Q software version 2.1.1 (QIAGEN) in a rotor gene Q (QIAGEN). A standard curve with different methylation percentages (0%, 25%, 50%, 75% y 100%) was made and triplicate samples were compared with standard (EpiTect) control DNA (QIAGEN). Samples of DNA without bisulfite treatment were used as negative control (Fig. 2).

2.5. RNA extraction, cDNA synthesis and real time- PCR

Total RNA from both plates of placental tissues was extracted with RNAeasy kit Columns (Lipid Tissue Mini Kit Qiagen) according to manufacturer's instructions and then frozen at −80 °C until cDNA synthesis. The RNA integrity for each sample was evaluated by 260/280 ratio of 1.7–1.9 and by electrophoresis in agarose gels. We used one μg of total mRNA for the synthesis of the cDNA with the M-MLV kit reverse transcriptase (RT, Promega, Wisconsin, USA) following manufacturer instructions. We quantified FOLR1 mRNA by the Eco qPCR System (Illumina San Diego, CA, U.S.A). Results were analyzed by the Eco real-time PCR System Software v4.1 (Illumina) and the relative quantification (QR) was calculated by the $-2^{\Delta\Delta}$CT method following the MIQE Guidelines [25]. Results were expressed in relation to the geometric mean expression of three of the most stable placental housekeeping genes (GAPDH, YWHAZ and β-actin). Primers for *FOLR1* are from Life Technologies (code: Hs 001124177_m1). Results were compared with those obtained in samples from term birth placentas.

2.6. Determination of folates and vitamin B12 in cord blood

Folate and vitamin B12 were determined in umbilical cord blood samples from newborns. After extraction, samples were centrifuged at 3000g for 10 min to isolate serum and then, aliquots were stored in polypropylene tubes at −80 °C. Total folates and vitamin B12 were determined by electrochemiluminiscence in an automated equipment (Cobas 411, Roche) using same brand chemicals. Analyses were performed in Vida Integra Laboratory (Santiago, Chile) certified by the Chilean Health Superintendence.

2.7. Statistical analysis

We employed Shapiro Wilk test to assess the normality of the variables and the nonparametric Kruskal Wallis followed by Mann Whitney tests to compare differences among groups. All results are expressed as median and range (P25-P75). Correlation analysis were performed by the Spearman test. Association analyses were obtained by linear regressions to estimate β coefficients with their respective intervals of

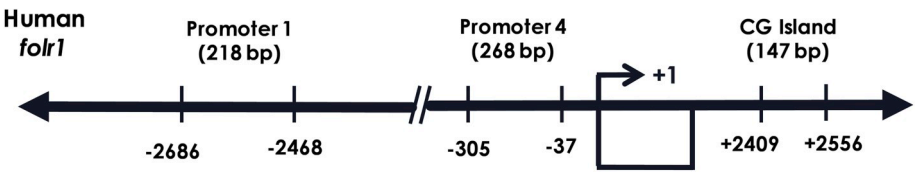

Fig. 1. Location of the CG island in the MethPrimer.
The human *FOLR1* gene scheme showing the two promoter regions (1 and 4) and the CG island of 147 bp located upstream the transcription initiation site.

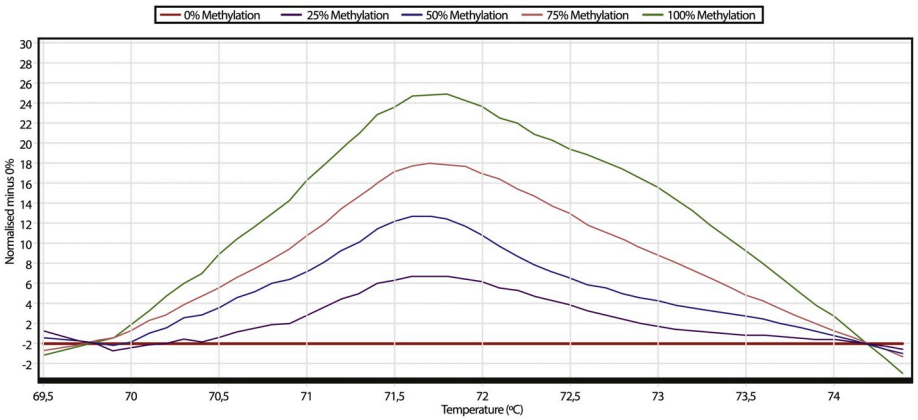

Fig. 2. HRM of samples with different methylation percentages.
Standard curves with different methylation percentage of bisulfite-treated samples.

confidence (CI). A value of $p \leq 0.05$ was considered significant. Statistical analyses were performed with the STATA Programme (StataCorp LLC) version 13.

3. Results

3.1. Characteristics of newborns and vitamin concentrations

Data of newborns are shown in Table 1. As expected, there are differences between both groups related to gestational age, placental weight and birth weight and height. Total folate concentrations in cord blood serum from preterm were higher than in term control newborns ($p < 0.05$). On the contrary, preterm newborns presented lower cord blood levels of vitamin B12 than the control group.

3.2. FOLR1 expression and methylation

The mRNA of FOLR1 in both, BP and CP of placenta samples, was lower in preterm compared to term group ($p < 0.05$) (Fig. 3). Methylation level in the *FOLR1* CpG island was higher in preterm compared to term group ($p < 0.05$) only in the CP. When comparing the methylation level of *FOLR1* between both plates within the same group, significant differences were only observed between CP and BP from the preterm group (Fig. 4).

Table 1
Anthropometric characteristics and vitamin concentrations.

Variable	Term	Preterm
Maternal age (years)	30 (22–38)	27 (19–40)
Gestational age (weeks)	39 (37–41)	35 (32-36)*
Birth weight (g)	3275 (2760–3650)	2240 (1490-3280)*
Birth height (cm)	49.8 (45.5–52)	45 (33-50) *
Placental weight (g)	582 (426–800)	518 (301-670)*
Gender (male/female)	9/7	12/11
Cord blood folate/nmol/L)	40.8 (32.9–66.3)	66.4 (41.2–90.4)*
Cord blood vitamin B12 (pmol/L)	307 (242.3–458.9)	209.7 (144.8–304.9)*

Values are expressed as median (min – max). Mann Whitney test, *$p \leq 0.05$.

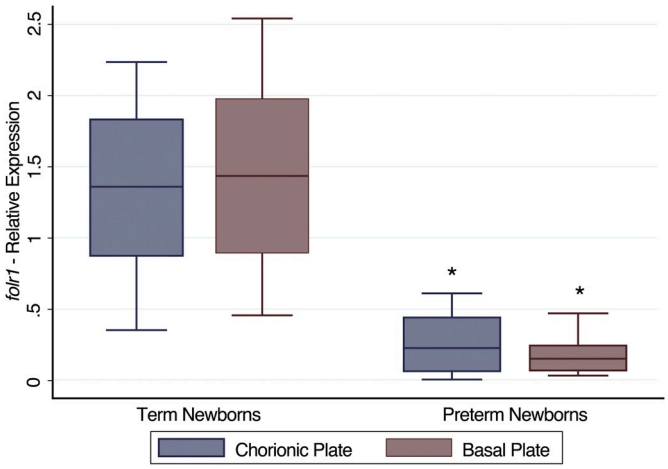

Fig. 3. Relative expression (mRNA) of *FOLR1* in BP and CP of human placentas.
The mRNA of *FOLR1* was determined by qPCR in relation to the geometric mean expression of 3 housekeeping genes (GAPDH, YWHAZ and β-actin) and expressed as median and 25-75th percentiles. Mann Whitney test, *$p < 0.05$, compared with their respective plate in term newborns.

3.3. Association between FOLR1 mRNA, methylation and folate/vitamin B12 levels in cord blood

We found an inverse association between *FOLR1* methylation level and the relative mRNA expression in the CP of the placental samples (Table 2; Spearman's Rho = - 0,526, p = 0,002). Also, a positive correlation between methylation level of *FOLR1* CpG island in CP and the folate/vitamin B12 ratio in cord blood (Spearman's Rho = 0,637, p = 0,019) was observed (Table 2). In the CP, a negative correlation of *FOLR1*-CpG island methylation was observed with gestational age and birth weight and height (Table 2). Results show that for each additional gestational week, the *FOLR1*- CpG island methylation may be reduced

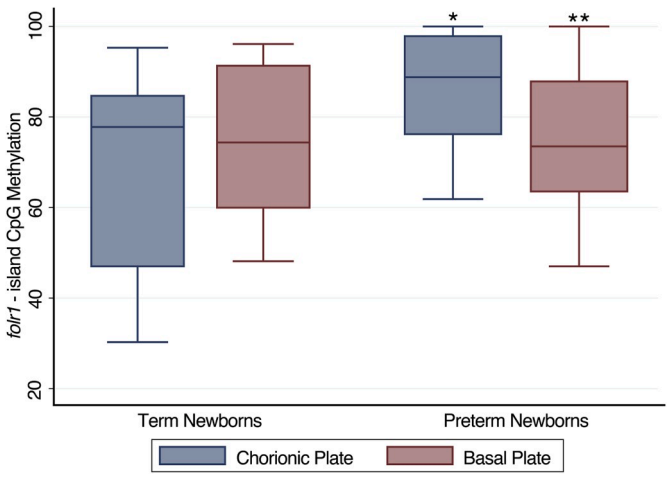

Fig. 4. Methylation of *FOLR1* CpG island in BP and CP of human placentas.
Methylation of *FOLR1* CpG island was determined by HRM post qPCR and expressed as median and 25-75th percentiles. Mann Whitney test, *p < 0.05, compared with CP in term newborns; **p ≤ 0.05, compared with CP of the same group.

Table 2
Correlations between *FOLR1*-CpG island methylation in human placentas.

Variable	Rho	Rho
	Chorionic Plate	Basal Plate
Gestational age (weeks)	−0.409 *	0.055
Birth weight (g)	−0.361*	−0.138
Birth height (cm)	−0.348 *	−0.150
Cord blood folate (nmol/L)	0.665 *	−0.539
Cord blood vitamin B12 (pmol/L)	−0.451	0.071
Folate/vitamin B12 ratio	0.637 *	−0.341
FOLR1 mRNA relative expression	−0.526 *	−0.152

Mann Whitney test, *p ≤ 0.05.

−3.56 units. Thus, in a crude model (not adjusted by weight or size), it would explain a 19.7% of the total variation in the methylation occurring in the specific gene area. However, when the model is adjusted for the anthropometric variables (weight and height), the methylation of the *FOLR1* CpG island explains a 22.2% of the total variability, reaching a borderline value of significance, possibly due to the low sample size (p = 0.054) (Table 3).

Table 3
Linear regression models.

Model	β coefficient (CI 95%)	R2	p
Crude model[a]	−3.56 (−6.08, −1.05)	0.197*	0.007
Model adjusted by birth weight	−0.80 (−6.22, 4.62)	0.209*	0.026
Model adjusted by birth height	−1.26 (−5.48,2.95)	0.220*	0.021
Model adjusted by birth weight and height	−0.88 (−6.37, 4.59)	0.222	0.054

Linear associations between CpG island methylation of *FOLR1* in chorionic plate and newborn variables at birth.
Values represent β coefficients (95% CI) of the gestational age as a predictor variable using CpG island methylation of *FOLR1* in chorionic plate as a response variable.
*p ≤ 0.05.
[a] Gestational age (weeks); birth weight (g); birth height (cm).

4. Discussion

Results obtained in this study show an increased methylation of *FOLR1* in preterm compared to term placentas, which is inversely related to the mRNA expression. Changes in the *FOLR1* methylation were only significant in the fetal side of the placenta (CP). This could be related to the different cellular types present in both areas of the placental tissue. The syncytiotrophoblasts (STB) are mainly present in the BP, while cytotrophoblasts (CTB) are mainly located in the CP of the placenta [26] and thus evidencing the morphological differences between both placental areas [27]. Moreover, when comparing cellular populations of CTB and STB, CTB showed more immunoreactivity for the DNA-methyltransferase 1 and 3a (DNMT1 y DNMT3a) whose functions are, respectively, to maintain the existing methylation pattern and to regulate *de novo* methylation, adding methyl groups to non-modified cytosines [26]. Likewise, methylation pattern present in cord blood from newborns registered specific CpG methylation, depending on the cell type, with significant changes in function of the gestational age [28]. This may suggest that the epigenetic pattern would be specific to the cell type and could be modified during the gestation period.

Although the receptors involved in the physiological transportation of the folates are located at the micro villus membrane and basal membrane the placenta, the measurement of FOLR1 in both plates separately, does not necessarily represent the physiological situation. Even so, we also determined FOLR1 expression in both placental plates, because there are antecedents indicating that gene expression may be dependent on the site where the sample is taken, due to the cellular heterogeneity within the tissue [26]. We found that in preterm placentas, FOLR1 is less expressed than in term placentas; this finding was observed in both plates.

However, in a previous study we found that protein levels of FOLR1 were higher in placentas associated to preterm than in term newborns, increasing the folate transport to the fetus; a finding evidenced by the higher folate concentrations in the preterm cord blood compared to term newborns [19]. These opposite results between mRNA expression and protein level may be explained by a “down-regulation” of FOLR1 mRNA through high folate levels, which in turn may alter the one carbon cycle, thus increasing the specific methylation level of the *FOLR1* gene leading to a lower gene expression. The lower levels of vitamin B12 observed in cord blood of preterm newborns could potentiate those effects.

Reports in the literature show discrepant results related to FOLR1 expression with pregnancy progression. Williams et al. [29], found a decreased mRNA expression of all folate transporters as the gestation progressed. However, those authors studied the FOLR1 expression in a wider range of gestational period (9, 12 and 39 weeks), unlike our study, where the samples of preterm placentas were collected between 32 and 36 weeks at birth. Different results were also reported by Solanky et al. [16], and more recently, by Mohanraj et al. [18], who found that FOLR1 expression remains unchanged from the first trimester of pregnancy until term. We propose here that, the lower expression of FOLR1 observed in our samples of preterm placentas would be a consequence of the increased methylation of the *FOLR1* gene that we found on the fetal side of the placenta, evidencing a gene specific epigenetic alteration associated to preterm pregnancies.

It is known that a hypomethylation in critical areas of the DNA leads to an increased expression of a specific gene, and, in the same way, a hypermethylation leads to a decreased gene expression [30,31]. In our study, FOLR1 expression showed a negative correlation with the placental CP methylation level (Table 2) in agreement with Farkas et al. [22], who reported and inverse association between gene methylation and expression for the placental *FOLR1* gene.

FOLR1 methylation and expression have also been studied in placental tissue with NTD and other birth defect-complicated pregnancies [18,22,29,32]. According to our knowledge, there is only one study in the literature that determines the methylation of *FOLR1* in placental tissue from NTD pregnancy; it shows that there were no changes

between NTD and controls [22]. In such study, the methylation level in all studied CpG sites of placental *FOLR1* presented a relatively high percentage of methylation (>50%), in agreement with the methylation level of *FOLR1* found in our study, despite different methods used in both studies to determine the methylation level (HRM vs pyrosequentiation) [22]. In relation to FOLR1 expression in placentas from NTD or other birth defects pregnancies, there are different results, showing an increased expression in NTD compared to the normal second-trimester placenta [18], or no effects between placental FOLR1 expression in pregnancies complicated with birth defects compared to controls [32]. Additionally, in preeclampsia, a lower placental FOLR1 expression, compared to normal pregnancies, has been reported [29].

In a previous study with full term pregnancies, we found a decreased FOLR1 expression in small and large compared to adequate for gestational age newborns [17], which suggests a relationship between FOLR1 expression and intrauterine growth.

Also, it is important to consider that some authors have found that folate transporters mRNA are susceptible to "up-regulation" under folate deficiency at intestinal level [33]; and similar mechanisms may be operating at placental level, thus justifying a decreased mRNA expression under high folate concentrations.

We found a positive association between the methylation level of *FOLR1* CpG in the placental CP and the folate/vitamin B12 ratio in cord blood (Table 2). This result is in the same line of evidence demonstrating an impact of dietary maternal use of methyl group donor on global and gene specific DNA methylation in newborns [34–36]. A dietary imbalance between vitamins of the B complex may affect the methyl donor availability and thus modifying the methylation of specific genes, as previously demonstrated [37].

The result that cord blood of preterm newborn presented higher folate and lower vitamin B12 concentrations than term newborns may be a consequence due to the impact of the application of a public policy of wheat flour fortification with folic acid initiated in Chile in 2000. Additionally, pregnant women are being supplemented with folic acid, according to the Ministry of Health recommendations, starting at initiation of conception [38,39]. Folate concentrations found in cord blood reflects folate maternal intake [5], so we can assume that during pregnancy, mothers are consuming enough amounts of folates (mainly as folic acid) which are being transported to the fetus.

Although some authors have found that placental folate transport and binding are not impaired in pregnancies complicated by fetal growth restriction [40], our results, along with others in the literature, suggest that maternal imbalance of folate and vitamin B12 levels are likely linked to suboptimal pregnancies, and related with complications during pregnancy. In a prospective observational cohort study with South Indian women, high folate and low vitamin B-12 intakes during pregnancy were associated with small-for-gestational age infants [37]. In addition, low vitamin B12 status in maternal plasma in early pregnancy was associated with a significant elevation of HOMA-IR and increased risk of insulin resistance in offspring by early school age [41]. Recently, a study analyzing cord blood folates and vitamin B12 among other metabolites, found that in women with preeclampsia, folate levels were higher and vitamin B12 lower compared to controls [42]. All these complications during pregnancy may be mediated through epigenetic alterations of specific genes in placenta and/or fetal tissues because of the imbalance between both vitamins.

In summary, we concluded that preterm pregnancies might be associated to the intake of unbalanced amounts of dietary B vitamins during pregnancy, altering fetal growth and development through mechanisms involving epigenetic changes of placental FOLR1, affecting their expression and, thus, establishing a major link between early environmental/nutritional exposure and increased risk of long term disease development in the offspring.

Declaration of competing interest

The authors declare no conflict of interest.

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