Preliminary Communication

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Epigenomics

Altered DNA methylation in human placenta after (suspected) preterm labor

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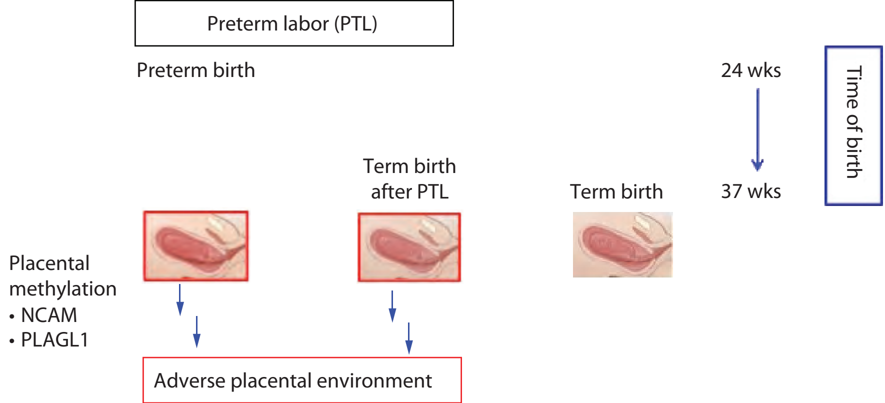
Aim: The aim of this study was to determine if alterations in DNA methylation in the human placenta would support suspected preterm labor as a pathologic insult associated with diminished placental health. **Methods:** We evaluated placental DNA methylation at seven *loci* differentially methylated in placental pathologies using targeted bisulfite sequencing, in placentas associated with preterm labor (term birth after suspected preterm labor [n = 15] and preterm birth [n = 15]), and controls (n = 15). **Results:** DNA methylation levels at the *NCAM1* and *PLAGL1 loci* in placentas associated with preterm labor did differ significantly ($p < 0.05$) from controls. **Discussion:** Specific alterations in methylation patterns indicative of an unfavourable placental environment are associated with preterm labor per se and not restricted to preterm birth.

Graphical abstract:



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The mammalian placenta orchestrates efficient exchange of nutrients, waste and gas between mother and fetus and contributes to the fetal environment through its endocrine functions [1]. Placental dysfunction underlies

pregnancy complications such as preeclampsia, gestational hypertension, small-for-gestational-age infants and spontaneous preterm birth. An imbalance of pro- and anti-angiogenic growth factors and deficient uteroplacental vascularization, as well as placental inflammation [2–4], represent pathological conditions that are common to the majority of pregnancy-related syndromes including preterm labor.

Preterm labor (PTL) is defined by increased uterine contractility with intact membranes requiring hospitalization and follow-up at <37 weeks of gestation, and affects 10–24% of pregnancies [5]. It is a frequent cause for hospital admission, and therefore of economical and practical relevance. PTL is deemed to be the common outcome of a variety of pathologies including infections, disruption of maternal–fetal tolerance, premature trophoblastic or decidual senescence, inflammation and a decline in progesterone action [5,6]. Not all cases of PTL end in preterm birth (PT). Spontaneously, or after acute intravenous tocolysis, actually in about 50% of cases birth occurs normally at term (≥37 weeks weeks). These latter cases are referred to as suspected preterm labor (SPL). While historically SPL has been considered neutral for posterior health, evidence is accumulating that risks are associated with SPL [7]. These include a higher incidence mall-for-gestational-age neonates compared with those born at term [8], increased frequency of neurodevelopmental and behavioral problems at preschool age [9], as well as retarded neurodevelopment [10]. In the latter study, no significant differences were found in any cognitive domain between children who were born late preterm and children who were born at term after suspected PTL (TBSPL).

In most human tissues, the vast majority of the genome is highly methylated (>70%) [11,12]. DNA methylation is among the epigenetic mechanisms that control gene expression and regulation, both during embryonic development [13,14] and in human pathologies [15,16]. Alterations in DNA methylation have been documented in human pathologies including human cancer [17,18] and placental pathologies that lead to reduced infant growth rate, pre-eclampsia and preterm delivery (PT) [19–21]. A continued examination of the relationship between epigenetic alterations in the placenta and placental physiopathology, developmental programming of the fetus and postnatal health will help clinical practice in the long-term.

In this manuscript, we analyzed DNA methylation, to determine whether alterations in human placenta may be used to compare SPL (resulting in either term [TB] or preterm [PT] births) with normal births. A variety of studies [22] has addressed alterations in the epigenome associated with PT and labor, invariably examining maternal or fetal tissues as opposed to placental tissue [23,24]. We are interested in placental pathologies, and placental epigenetic markers for PTL have not been defined so far. Therefore, we selected a set of *loci* whose methylation levels are reportedly altered in placental pathologies, in the hope of describing alterations specific to PTL as opposed to PT. In particular, we selected inflammation-related *loci* whose placental methylation levels are altered in preeclampsia (*NCAM1* and *TNF* or *TNFA*) [25,26] or under conditions associated with placental stress such as low oxygen conditions (*CFB* and *ZNF217)* [25]. Both the *FLT1* [27] and *VEGFA* genes were included based on the importance of angiogenesis in placental pathologies [3,5]. Genomic Imprinting describes the determination of gene dosis (and ultimately expression levels) based on parental-origin-specific methylation patterns [28,29]. As a group, mammalian imprinted genes play an important role in the placenta by regulating fetal and placental growth and development [30,31], and deregulation of a variety of imprinted genes contributes to placental pathologies. The imprinted gene *PLAGL1* encodes a paternally expressed transcription factor (also called *Zac1* in the mouse and *LOT1* in human) that binds to target genes through zinc-finger domains [32,33]. As altered placental expression of *PLAGL1* has been directly implicated in IUGR (Intrauterine growth restriction) [34], this locus was included in our analysis. We have used bisulfite-genomic DNA sequencing to compare methylation patterns at these selected loci between term born, PT and term birth after suspected PTL.

Materials & methods

Ethics statement

The local ethics committee (CEICA) approved the study protocol (CP – CI PI13/0162 08/01/2014), and parents provided written informed consent (protocolo y información para el paciente v2 de 29/12/2013).

Placental DNA samples & genomic loci analyzed

Samples analyzed represent a subset of a prospective cohort study of preterm (26–36 weeks) or term (≥37 weeks) delivery pregnancies after suspected PTL. Suspected PTL was defined by uterine contractions, ultrasound cervical length <25 mm in the presence of intact membranes. For more information we refer to the original description of the cohort [35]. Subjects from each condition were selected at random from the original groups described in [35], complying with two more requisites: we included the maximum number available of cases defined as 'inflammation'

in the hope of extracting useful data under these conditions, and we made sure that the average levels of expression in the selected group were similar to those reported in the cohort [35].

Placental tissue was sampled from chorionic villus sites and stored in RNA™ later as described [35]. To extract DNA, tissue was homogenized in 1 ml of genomic lysis buffer (30 mM Tris-HCl pH 8.5, 40% [m/v] guanidinium chloride, 30 mM ethylenediaminetetraacetic acid [EDTA] pH 8), the resulting supernatant was incubated overnight with 1 mAU protease (QIAGEN, Hilden, Germany) at 65°C. Cell debris and other small particles were removed by centrifugation at full speed for 2 min at room temperature (RT) and the homogenate was frozen at -80°C until further use. Genomic DNA was isolated by isopropanol precipitation according to standard protocols, re-suspended in 100 μl of TE buffer (10 mM Tris-Cl pH 8, 1 mM EDTA pH 8) and stored at -80°C. To extract DNA, tissue was homogenized in 1 ml of genomic lysis buffer (30 mM Tris-HCl pH 8.5, 40% [m/v] guanidinium chloride, 30 mM EDTA pH 8) and, the resulting supernatant was incubated overnight with 1 mAU protease (QIAGEN) at 65 ºC. Cell debris and other small particles were removed by centrifugation at full speed for 2 min at RT and the homogenate was frozen at -80ºC until further use. Genomic DNA was isolated by isopropanol precipitation according to standard protocols, resuspended in 100 μl of TE buffer (10 mM Tris-Cl pH 8, 1 mM EDTA pH 8) and stored at -80ºC. The amplicons analyzed for methylation levels have been previously described in the following reports: FLT1 [36]; VEGFA [36]; ZNF217 [25]; PLAGL1 [37]; CFB [25]; NCAM1 [26]; TNF [26] (Gene nomenclature as approved by HUGO Gene Nomenclature Committee [HGNC]). Amplicons were selected in the 150–250 bp size range to facilitate posterior sequencing (see below). Genomic localization (Homo sapiens GRch 38.97), and other details regarding the amplicons employed are listed in Table 2, each amplicon contained a range of 2–22 CpGs. The locus-specific sequences of primers are listed in Table 3.

Methylation analysis

Genomic DNA was bisulfite converted using the EZ DNA methylation kit (Zymo Research Corp., CA, USA) following the manufacturer's instructions. As a measure of successful conversion, overall percentages of non-CpG methylation varied from 0.03 to 0.06 % among all loci and samples studied. Target regions (Table 2) were amplified using tailed oligos that consist of a unique amplicon-specific part, fused to a 5′-tail comprising sequences necessary for library preparation and sequencing reactions, and a 10 bp multiplex identifier (MID) to identify each sample. Amplification products were purified from agarose gels, titrated, diluted, pooled, subjected to emulsion-PCR. The resulting amplicon-specific libraries were subjected to Illumina sequencing in MiSeq (2 × 250 reads). The sequencing run yielded over 840000 filtered, quality reads, an average of about 1800 reads per amplicon per sample (range 500 to 5000). Bisulfite conversion, amplification of target sequences and sequencing were carried out at Fundación Parque Científico de Madrid (FPCM), c/Faraday 7, Madrid, Spain. Reads obtained were filtered and sorted according to their MID and the reference sequence. Alignments and calculation of the percentage methylation have subsequently been performed using the freely available software Bismark [38]. The percentage of methylation per sample within each CpG was calculated as the percentage C/C+T, the mean value of all CpG dinucleotides per amplicon was calculated to represent the methylation value of a particular locus.

Gene expression analysis

Tissue homogenisation, RNA extraction, reverse transcription (RT) and quantitative polymerase chain reaction (qPCR) were carried out as described [35], on the same biopsies analyzed for DNA methylation (see above). Primers for detection of *PLAGL1* expression have been reported before [34]. TaqMan® qPCR assays (Applied Biosystems, CA, USA) used for all other markers are listed in Table 3. *RPL19* and *SDHA* were used as reference genes [34,39].

Expression suite (Thermo Fisher Scientific, MA, USA) was used to calculate ΔCt values with respect to the average of the cycle threshold (Ct) values of the *RPL19* and *SDHA* reference genes. Data were further recalculated as 2-ΔΔCt, with respect to the mean ΔCt value of the samples in the control group as described [40]. The resulting numbers are listed in the Figures and represent fold change in expression.

Statistical analysis

Normal distributions were assessed using Kolmogorov–Smirnov tests. Mann–Whitney or Student's t-test was used to compare cases and controls. Analysis of variance (ANOVA) or Kruskal Wallis tests were used to compare the three groups. Chi-square or Fisher tests were used to analyse categorical variables. Post hoc Bonferroni tests were applied to compare multiple groups. Data are presented as mean ± standard deviation (SD). Two-sided p-values

< 0.05 were considered statistically significant. SPSS Software (v. 20, SPSS Inc., IL, USA) was used for statistical analyses.

Results

Placental DNA methylation

To study potential epigenetic alterations associated with PTL, we analyzed in this study a subset of the 104 placentas from the cohort of 132 patients examined in another study recently published by our group [35]. We included 15 from each group: PT, delivery at TBSPL and normal term delivery (control [con]). Groups were chosen with the intention to both include a maximum number of cases with sterile inflammation (see below), and maintain median expression levels of angiogenic factors similar to the original groups [35]. Maternal sociodemographic characteristics and perinatal data are shown in Table 1. There were no significant differences among groups in the number of smokers included, sex of the infants or maternal age. Preterm infants had significantly lower birth weights, earlier gestational ages at delivery and, were admitted to the neonatal care unit at a significantly higher rate than term-born infants. Moreover, significantly different from the normal term delivery group, women in PTL groups (both PT birth and TB) had a lower maternal body mass index, a lower frequency of spontaneous labor and higher rates of PT in previous pregnancies.

To study potential epigenetic alterations in the placenta associated with PTL, DNA methylation levels of selected *loci* (Table 2) were compared between PT, TBSPL and Con groups. Candidate targets were selected based on reports describing differential methylation in human placenta tissue associated with preeclampsia or placental pathology-related processes (see Introduction). We applied massive parralel sequencing of PCR products amplified from bisulfite-treated DNA to determine the DNA methylation status of the selected DNA regions. Representative results for the *PLAGL1*, *TNF* and *CFB loci* are shown in Figure 1. Data for the four remaining *loci* can be viewed in Supplementary Figure 1. While the mean methylation level per CpG varied across the *loci*, the mean methylation level per CpG in each group (PT, TBSPL and Con) was remarkably consistent (higher in Con for *PLAGL1*; lower in Con for *CFB*; only small changes in *TNF)* across the amplicons. Except for the *FLT1* locus, no alterations were detected in individual CpGs that were different from those calculated across amplicons.

Table 1. Maternal sociodemographic characteristics and perinatal data.

	Preterm delivery (n = 15)	Term delivery after suspected preterm labor (n = 15)	Term delivery control (n = 15)	p-value
Maternal age, mean (SD)	27.4 (6.1)	31.2 (6.5)	31.03 (4.7)	0.149
Maternal body mass index, kg/m^2, mean (SD)	23.8 (4.3)	21.4 (2.6)	26.7 (4.5)	0.006
Nulliparity, n (%)	9 (60)	8 (53.3)	12 (80)	0.283
Caucasian, n (%)	10 (66.7)	15 (100)	12 (80)	0.340
Smokers, n (%)	1 (6.6)	1 (6.6)	3 (20)	0.429
Preeclampsia, n (%)	1 (6.7)	0 (0)	0 (0)	0.306
Infant sex, male, n (%)	7 (46.7)	11 (73.3)	8 (53.3)	0.306
Gestational age at birth, days, mean (SD)	243 (18.6)	276 (10.1)	275 (7.7)	0.001
Previous preterm delivery, n (%)	2 (13.3)	4 (26.7)	1 (6.6)	0.023
Birth weight, g, mean (SD)	2240 (482.3)	3167 (344.7)	3282 (398.0)	0.001
Cesarean section, n (%)	2 (13.3)	4 (26.7)	8 (53.3)	0.055
Labor[†], n (%)	15 (100)	11 (73.3)	8 (53.3)	0.012
Cervical ripening[‡], n (%)	0 (0.0)	4 (26.7)	3 (20)	0.111
Antenatal steroids, n (%)	5 (33.3)	12 (80)	0 (0.0)	0.001
Placental inflammation[§], n (%)	5 (33.3)	3 (20)	0 (0.0)	0.056
Apgar at 5 min <7, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	1
Neonatal care unit admission, n (%)	9 (60)	1 (6.7)	1 (6.7)	0.001
Umbilical artery pH <7.15, n (%)	0 (0.0)	1 (8.3)	0 (0.0)	0.675

[†] Spontaneous labor.
[‡] Prostaglandin E2.
[§] Placental expression level of both *IL6* and *TNFA* >1.5 multiples of the median. Data were analysed using ANOVA (continuous variables) and either Fisher or Chi-square tests (categorical variables).
ANOVA: Analysis of variance; SD: Standard deviation.

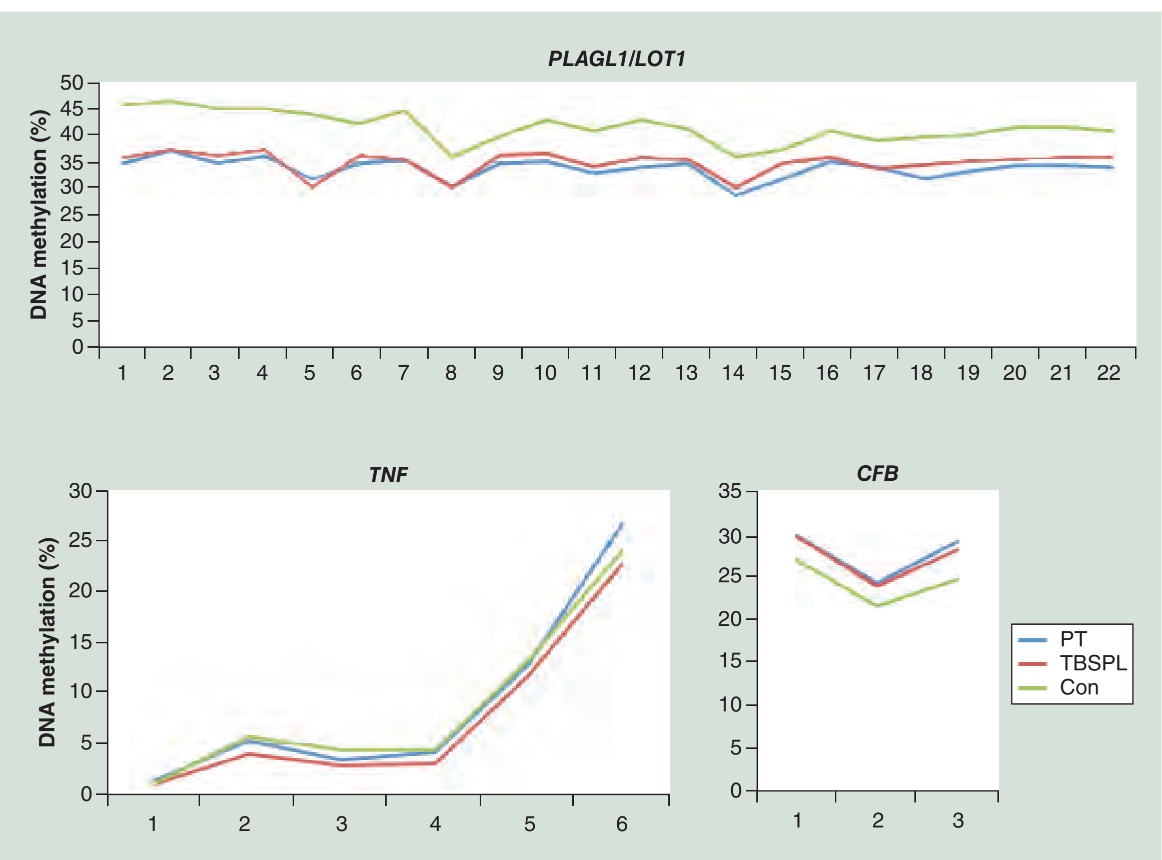

Figure 1. Placental DNA methylation at consecutive CpG dinucleotides across the genomic regions analyzed for the *LOT1/PLAGL1*, *TNF* and *CBF* loci as indicated. Mean DNA methylation at each CpG site in control samples (n = 15, green), or samples defined by preterm labor and term born (TBSPL, n = 15, red) and born preterm (PT, n = 15, blue), respectively. The percentage methylation is indicated on the vertical axis, consecutively numbered CpGs on the horizontal axis.
Con: Control; PT: Preterm birth; TBSPL: Term birth after suspected preterm labor.

Table 2. Amplicons for methylation sequencing assays.

Target	Chromosomal location	Gene region	Amplicon size	Reference	N° CpGs
VEGFA	6: 43769653	gene promoter	132	Kim *et al.*, 2012	7
FLT1	13: 28495234	gene promoter	195	Kim *et al.*, 2012	21
TNF	6: 31575675	promoter 001 transcript	177	Anton *et al.*, 2014	6
CFB	6: 31945473		236	Yuen *et al.*, 2013	3
NCAM1	11: 113276368	1 kb down-stream of translation STOP codon	154	Anton *et al.*, 2014	2
PLAGL1	6: 144007815	intron 1-2	177	Poole *et al.*, 2013	22
ZNF217	20: 53583123	promoter 201 transcript	277	Yuen *et al.*, 2013	5

Descriptions of amplicons produced for methylation sequencing assays, genomic localization is listed according to genome build Homo sapiens GRch 38.97.

Expression analysis:

Among the *loci* analyzed, methylation levels varied considerably (Table 4). In both normal placentas & in cases of PTL, CpG sites across the *FLT1* & *VEGFA* promoter regions were largely unmethylated (0–0.6 % methylation). By contrast, methylation in the *NCAM* gene amounted to 89% (92–96% in the first CpG), indicating almost complete methylation.

Table 3. Primers used.

Forward	Reverse	Ref.
(A) Methylation analysis:		
NCAM1	5′-TATTTTTGTGTTTTTTTGGGGGTTAGATTA	
	5′-CCCAACTATACAATCTTCTCTACTTCAT	
TNF	5′-AAACAAATTCTCTTCCTCTCACATACT	
	5′-GGAGAAGAGGTTGAGGAATAAGT	
PLAGL1	5′-GAGGAGGGTGTGTTTTTGT	
	5′-AATCTATAAACCTCATACCAAATAAAC	
VEGFA	5′-TAGGGAAGTTGGGTGAATGGA	
	5′-TCCTAAAATAACCCCTAACCTTCT	
FLT1	5′-ATGGGTAGGAGGAGGGGTAA	
	5′-TCCCCACCTACCCTCTTCTT	
CFB	5′-GGGTTTTTAGGATGTTAGAGGT	
	5′-CAACCCTACCTAACCTCCAAATAA	
ZNF217	5′-GGATTTTAATTGGATGAAATTTTG	
	5′-CAAAAAATCAATCCCAACAACCTA	
(B)		
PLAGL1 primers		[28]
F	5′ GTGAGGAGTGTGGGAAGAAG	
R	5′ GAGGTGGTCCAGTAGCACCTC	
Marker	TaqMan® assay	
NCAM1	Hs00941830_m1	
HSDA	Hs00188166_m1	
RPL19	Hs02338565_gH	

Table 4. Altered methylation after suspected preterm labor.

	PTL	Controls	p*	TBSPL	PT	Con	p-value
	n = 30	n = 15		n = 15	n = 15	n = 15	
VEGFA	0.3 (0.3)	0.3 (0.3)	0.540	0.3 (0.3)	0.3 (0.3)	0.3 (0.3)	0.786
FLT1	0.4 (0.5)	0.4 (0.5)	0.538	0.4 (0.5)	0.4 (0.5)	0.4 (0.5)	0.601
TNF	8.3 (9.0)	8.8 (8.8)	0.547	9.0 (9.7)	7.6 (8.3)	8.8 (8.8)	0.326
CFB	27.5 (6.7)	24.4 (5.8)	0.051	27.2 (5.8)	27.7 (7.5)	24.4 (5.8)	0.259
NCAM1	83.8 (12.4)	86.9 (9.5)	0.009	82.0 (13.9)	85.5 (10.7)	86.9 (9.5)	0.001
PLAGL1	34.3 (8.1)	41.7 (9.3)	0.006	35.2 (7.1)	33.9 (9.0)	41.7 (9.3)	0.021
ZNF217	56.6 (25.0)	55.3 (21.0)	0.508	57.1 (25.3)	56.1 (24.9)	55.3 (21.0)	0.729

Placental DNA methylation of selected genes. DNA methylation (%) across the amplicons indicated in Table 2. Methylation values (mean +/− SD) for each locus in placental samples:
Left panel: from controls (n = 15) and combined preterm labor samples (PTL; n = 30).
† p-values have been calculated using Student's t-test.
Right panel: from controls (n = 15), term birth after suspected preterm labor (n = 15), and preterm birth samples (n = 15).
‡ p-values were calculated using one-way analysis of variance.
Con: Control; PT: Preterm birth; PTL: Combined preterm labor samples; SD: Standard deviation; TBSPL: Term birth after suspected preterm labor.

Differential placental DNA Methylation in PTL

Out of the genomic regions selected, several of the *loci* analyzed displayed differential DNA methylation in the cases of PTL (PT and TBSPL combined) compared with controls (Table 4). Although we detected no differences between the two groups in the *TNF*, *FLT1, VEGF* and *ZNF217* genes, a trend toward higher methylation in the *CFB* gene (p = 0.051), as well as significantly lower methylation in the *NCAM1* and *PLAGL1 loci* was associated with PTL (Table 4); $p < 0.05$ (Student's t-test).

As the PTL group is a mixture of samples taken after either PT or term birth (TBSPL), the differences observed may be the result of differences in gestational age. As the latter is a variable that cannot be adjusted or controlled for in the PT group, we compared the three groups to identify alterations not limited to PT. Alterations in *CFB*, *NCAM1* and *PLAGL1* methylation trended similarly in both the PT and TBSPL groups, compared with the term

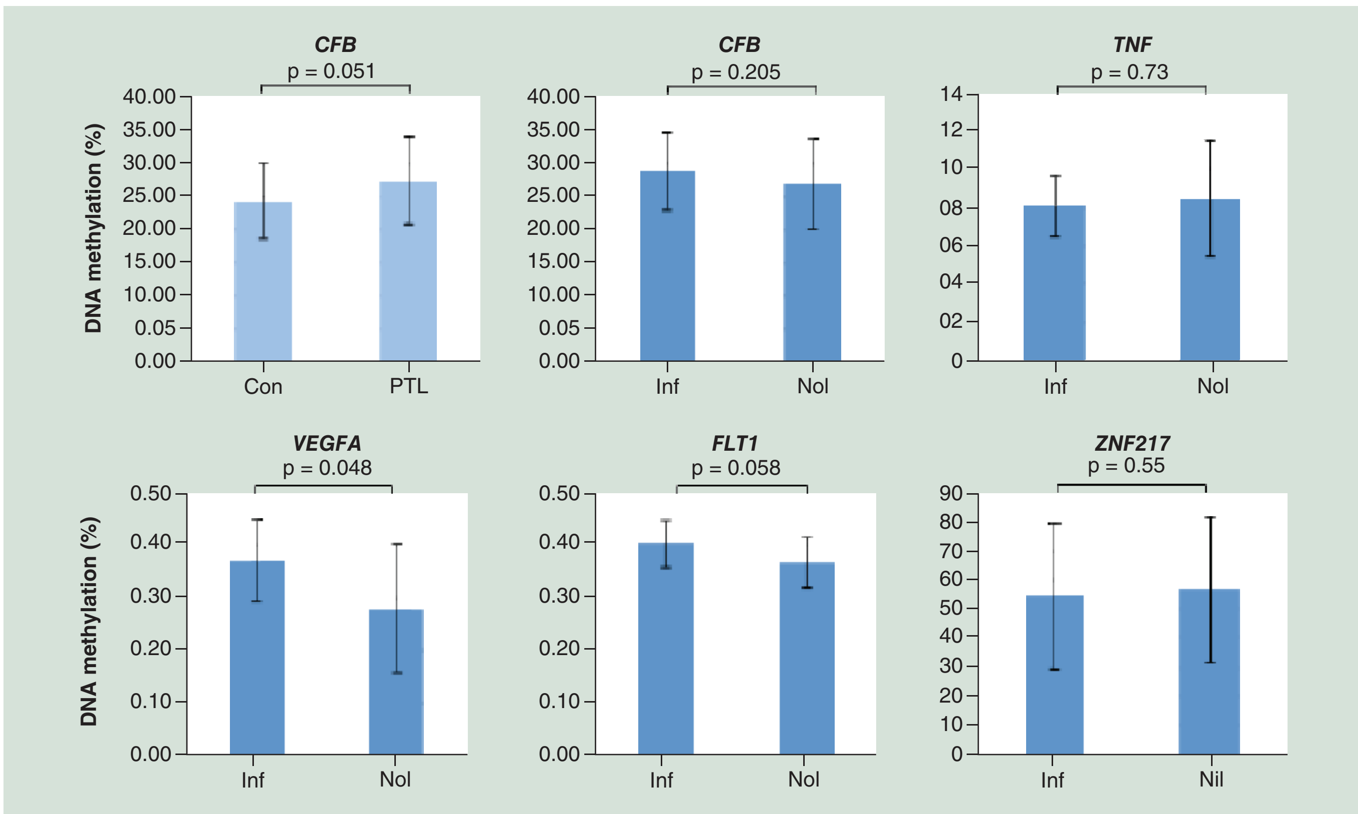

Figure 2. DNA methylation at CpG sites within the FLT1, VEGFA, TNF and CBF loci.
Placentas showing Inf (n = 8), all corresponding to the preterm labor group, are compared with NoI (n = 37), except for the CFB panel to the top left, which compares control placentas (n = 15) to placentas from combined suspected PTL pregnancies (n = 30). Mean methylation (%) values +/- SD at each locus were calculated across the region analyzed. Data were analyzed using using Student's t-test.
Con: Control; Inf: Inflammation; NoI: Placentas without inflammation; PTL: Preterm labor; SD: Standard deviation.

delivery control group (Table 4, right panel). Statistical analysis showed significant differences among the three groups in methylation levels at the *NCAM1* and *PLAGL1 loci* (p = 0.001 and 0.021, respectively). To further investigate differences between groups, we performed pair-wise comparisons. For several *loci*, no differences (p = 1) were detected between the PT and TBSPL groups (*CBF, PLAGL1, VEGF, ZNF217 and FLT1*). Methylation at the *PLAGL1* locus was altered in both the PT and TBSPL groups compared with the term delivery control group, although statistical significance was only reached for the PT vs. Con difference (see below). We conclude that at several *loci*, alterations in placental DNA methylation trended similarly in both the PT and TBSPL group, and therefore appear independent of gestational age.

Placental DNA methylation associated with Inflammation

Inflammation (in the absence of apparent infection) is a well-established cause of about 14% of PTL cases [41] and can arbitrarily be defined by increased placental levels of both *IL6* and *TNFA* [35]. We therefore assessed whether inflammation was associated with altered DNA methylation (Supplementary Table 1). No differences were detected for most *loci* including *FLT1*, *PLAGL1*, *TNF* and *ZNF217* (Figure 2 & Figure 4B). Although sample size in the inflammation group was small (n = 8), methylation at several *loci* was altered compared with the combined samples without inflammation (n = 37). A slight (nonsignificant) increase was detected in the *CFB lococ* in the inflammation group (Figure 2), in contrast to a significant increase in *CFB* methylation in the PTL group (Figure 2; panel CBF Controls vs PTL). A marginally significant change in DNA methylation associated with inflammation was observed in the *FLT1* promoter (Figure 2). Apart from these trends, significant decreases in DNA methylation as a result of inflammation occurred in the *VEGFA* (Figure 2) and *NCAM1 loci* (Figure 3).

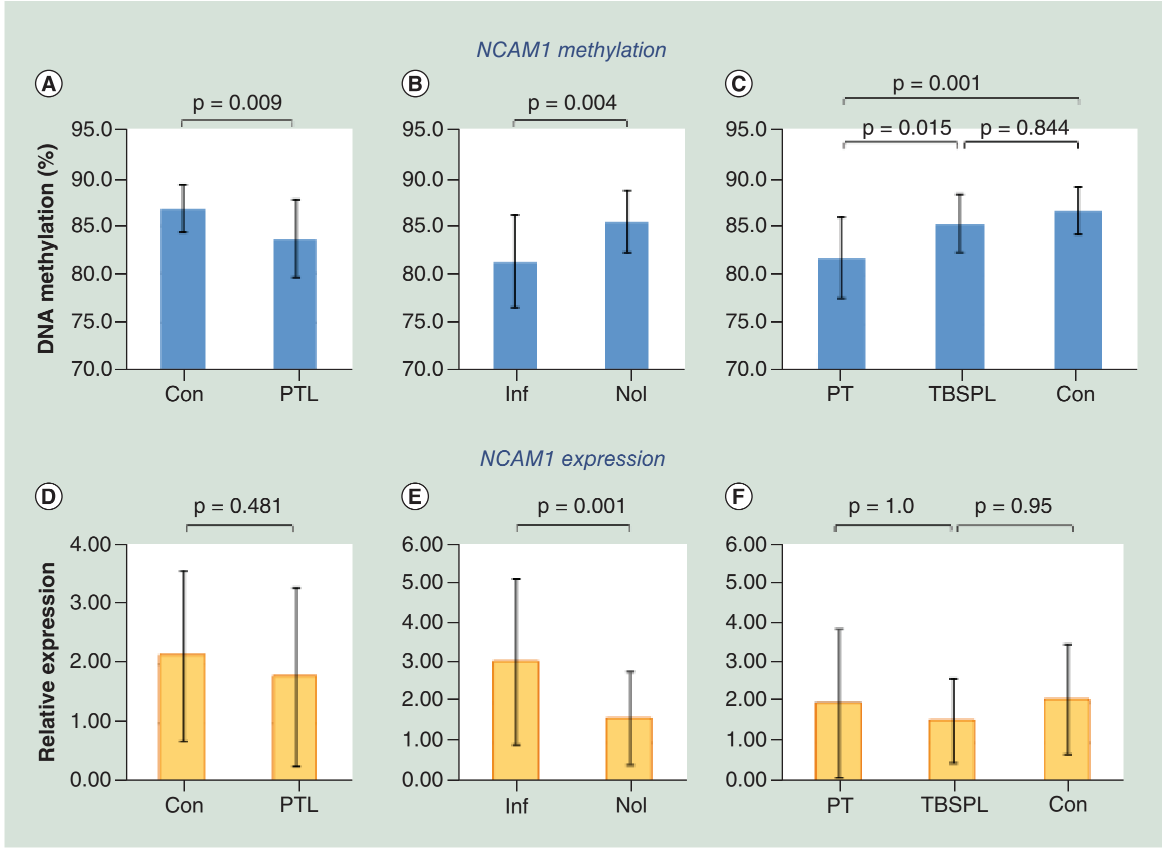

Figure 3. DNA methylation levels and mRNA expression levels of the *NCAM1* gene in placental samples. Control (n = 15) and combined preterm labor (PTL; n = 30) placentas were compared, as well as cases of Inf (n = 8) versus placentas NoI (n = 37) and Con (n = 15), TBSPL (n = 15) and PT (n = 15). **Top (A–C):** Mean placental DNA methylation at CpG sites downstream of the *NCAM1* locus (see Table 1) was calculated based on average CpG methylation measured by NGS of PCR products amplified from bisulfite-treated DNA. Data are shown as mean (+/- SD). Data were analyzed using Student's t-test (2 groups) or ANOVA (three groups). **Bottom (D–F):** mRNA levels were measured by qPCR using *RPL19* and *SDHA* as reference genes. Gene expression data are represented as mean value of each group +/- SD. ANOVA: Analysis of variance; Con: Control; Inf: Inflammation; NGS: Next-generation sequencing; NoI: Placentas without inflammation; PT: Preterm birth; PTL: Preterm labor; SD: Standard deviation; TBSPL: Term born after suspected preterm labor.

Increased NCAM1 expression is associated placental inflammation

To establish the functional significance of the observed changes in DNA methylation, gene expression levels of the *NCAM1*, *CFB* and *PLAGL1* genes were analyzed using qRT-PCR in the 45 placentas under examination. At the *NCAM1* locus, we had detected significantly decreased levels of methylation associated with PTL (Figure 3A), which was significantly associated with inflammation (Figure 3B) and rather specific for the PT group (Figure 3C, p = 0.001). Pair-wise comparisons were performed to investigate differences in *NCAM1* mRNA expression levels between groups. No differences were observed between the PTL and control groups (Figure 3D). Moreover, in contrast to the methylation levels, the expression level of *NCAM1* was not significantly different between the PT, TBSPL and Con groups (Figure 3F). However, increased *NCAM1* mRNA expression was clearly associated with inflammation (Inf), as relative expression was 3.08 and 1.63 in Inf versus NoI, respectively (Figure 3E). We identify epigenetic and transcriptional alterations in the *NCAM1* locus in cases of inflammation-related PTL.

The association of PLAGL1 with PTL

We had detected significantly decreased methylation levels at the *PLAGL1* locus associated with PTL (Figure 4A). Decreased methylation was not restricted to the PT group, but was observed to a different degree in both PT and TBSPL groups (Figure 4C). In agreement with the methylation data, expression of *PLAGL1* mRNA was about 2.5-fold increased in the combined PTL samples compared with controls (Figure 4D). Surprisingly, the *PLAGL1* mRNA

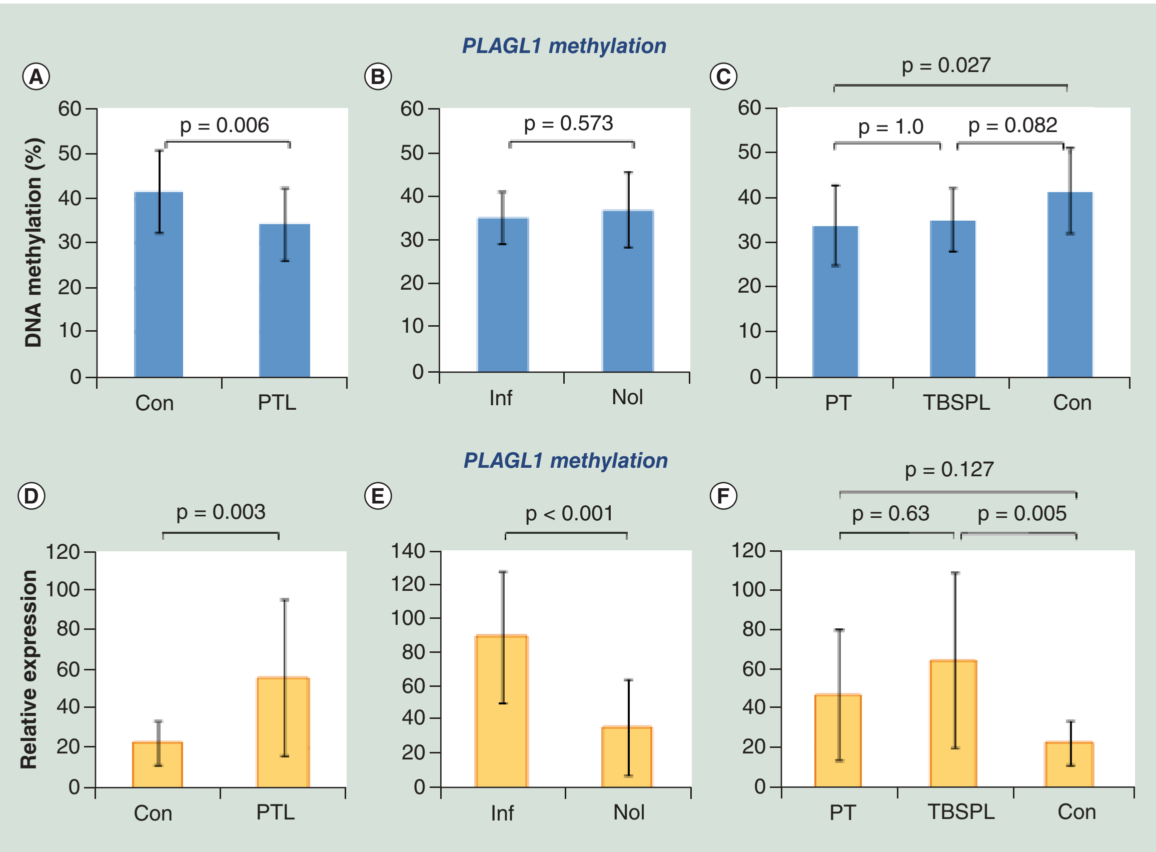

Figure 4. Changes in methylation status are associated with altered mRNA expression of *LOT1*/*PLAGL1*. As Figure 3, except for CpG sites within the first intron of the *PLAGL1*/*LOT1* gene.
Con: Control; Inf: Inflammation; NoI: Placentas without inflammation; PT: Preterm birth; PTL: Preterm labor; TBSPL: Term born after suspected preterm labor.

level was significantly higher in the inflammation (Inf) group, compared with the samples without inflammation (NoI), (relative expression was 89.39 and 35.68 in Inf vs NoI groups, respectively) (Figure 4E), as opposed to unaltered methylation (Figure 4B). The latter observation suggests dual regulation of *PLAGL1* mRNA levels by inflammation-dependent and -independent mechanisms of PTL. Pair-wise comparisons to investigate differences between groups (Figure 3F) showed that he expression levels of *PLAGL1* trended toward an increase in both PT and TBSPL groups, compared with the term delivery control group (p = 0.127 and 0.005, respectively). In this preliminary study, decreased placental DNA methylation and increased mRNA expression of *PLAGL1* was associated with PTL.

Discussion

Altered methylation is associated with PTL

We studied genomic methylation in placental DNA obtained from cases of PTL. Candidate genes were selected based on altered methylation in processes related to placental pathologies. The bisulfate-sequencing approach employed yielded data of high confidence in terms of methylation levels at each individual CpG analyzed. We found significant differences between cases of PTL and controls for methylation at the *NCAM1* and *PLAGL1* loci. Comparing Con, PT and TBSPT groups, we found no differences whatsoever in the *ZNF217* and *VEGFA* loci. In contrast to a reported decrease in global DNA methylation levels in preterm deliveries [42], we describe a trend toward increased methylation at the *CFB* locus (Table 4). At several *loci*, alterations in placental DNA methylation trended similarly in both the PT and TBSPL group, and were not limited to the PT group. We therefore believe these alterations are related to PTL and relatively independent of gestational age.

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It has been widely believed, that resolution of an episode of PTL has no secondary effects and does not cause long-term effects for the fetus, neonate or infant if birth occurs at term [43]. The present data on methylation patterns support an association between specific placental alterations and PTL. We postulate that the underlying placental pathology may ultimately pose a short-term or future health risk. In combination of previous reports on adverse effects on neurodevelopment [10] and placental gene expression [35], these results suggest that some of the biological mechanisms underlying PTL similarly affect both preterm and term-born infants. Recently, large scale studies have become available, which apply genome-wide technology to maternal or cord blood samples (and tissues) in order to study potential biomarkers and predictors of PT [23,24,44]. However, such information is not yet available for placenta. We hope that the potential epigenetic differences in the placenta related to PTL we describe here may stimulate future efforts toward defining the placental epigenome and transcriptome of PTL.

Limitations of the study

Despite identifying changes that support the hypothesis that alterations may result from PTL independent of the time of birth, the present study also has limitations. First and foremost, the sample size is small. The analysis presented would certainly benefit from increased sample size, both with respect to PTL groups, as to the presence of inflammation. Moreover, the potential influence of gender, maternal body mass index, labor and administration of antenatal steroids on DNA methylation levels cannot be excluded. While TBSPL and control samples are age-matched, by definition the PT samples are not. As a result, differences between PT and control samples may be related to gestational age. Furthermore, at present we cannot exclude that altered DNA methylation levels are a function of the proportion of different cell types present in the samples. As we tested methylation at *loci* whose selection was based on alterations in placental pathologies different from PTL, our study was not directed at identifying genes or *loci* that are directly involved in PTL. The differences in methylation presented in the *NCAM1* and *PLAGL1* genes are purely correlative as of now. Whether altered epigenetic and transcriptional control of these genes underlie pregnancy complications and PTL remains to be established.

Our work provides a starting point for further analysis aimed at understanding both the causes of PTL and the influence of PTL-associated changes on adverse intrauterine environment and posterior health.

Physiopathological implications of altered methylation

As disequilibrated immune responses are frequently postulated as primary initiators of the multiple subsequent pathways leading to placental pathologies [45], we analyzed placental methylation at several loci encoding components. No relationship was evident between methylation at the *TNF* locus and either PTL or inflammation. Increased expression of *TNFA* in placental inflammation apparently is independent of methylation levels of the *TNF* promoter. A significant increase in placental methylation was found in the *CFB* locus (Table 4), which in turn was independent of inflammation (Supplementary Table 1). To the best of our knowledge, this is the first report suggesting that the link between *CFB* and PTL merits further investigation.

Inflammation has been identified as a cause of about 14% of PTL cases [41,43]. It is therefore no surprise that we found altered methylation that was strictly associated with inflammation, as was the case for the *NCAM1* locus (Figures 2 & 3). In addition to a significant decrease in methylation in PTL (Figure 3A), both methylation (Figure 3B) and expression (Figure 3E) of *NCAM1* are significantly altered in case of inflammation. Moreover, as expected, decreased methylation corresponds with increased expression, suggesting functional significance. As NCAM1/CD56$^+$ expression is not normally detected in trophoblasts [46], we suggest the signals may correspond to either resident fetal macrophages (i.e., Hofbauer cells) [47], or infiltrating maternal cells, in other words, macrophages or natural killer (NK) cells. Infiltration of macrophages in the trophoblast layer has been reported in a case of spontaneous abortion [48].

In line with the importance of defective angiogenesis to placental pathologies including PT [5], we studied *VEGFA* and *FLT1* promoter methylation. We report low and PTL-independent overall methylation levels at both the *VEGFA* and *FLT1 loci* similar to a previous report in the latter case [27]. In the context of the extremely low level of *VEGFA* promoter methylation, a significant alteration in a small subset of cells may be relevant and drive altered expression levels.

PLAGL1 methylation is very similar between both PTL groups (Figure 4) and (significantly) lower in both PTL groups compared with normal births. In addition to altered methylation, increased *PLAGL1* expression levels are also associated with PTL (Table 4; Figure 4D). Methylation of this locus is lower in both PTL groups compared with controls. While altered methylation of PLAGL1 has been previously associated with imprinting disorders [49]

and preeclampsia [34], we describe here for the first time its association with PTL. Moreover, altered methylation is accompanied with increased gene expression of this locus (Figure 4D–E). In mouse models, *PLAGL1* has been described to play a central role in regulation of a network of imprinted genes, both in embryos [32] and in a neuroblastoma cell line [33]. The network controls cell cycle exit [33] and endocrine lineages [31]. Whether alterations in these pathways and functions may also affect placental cell types and contribute directly or indirectly to PTL remains to be established.

Conclusion

Based on the determination of DNA methylation levels at selected *loci* using targeted bisulfite sequencing, we analyzed differences between PTL and controls. DNA methylation levels of the *NCAM1* and *PLAGL1 loci* in placentas associated with PTL did differ significantly from controls. In placentas with inflammation, methylation levels were significantly altered at the *NCAM1* locus. The alterations associated with PTL we describe may be indicative of placental pathology associated with PTL, which is independent of the time of birth (term or preterm).

Future perspective

PTL is deemed to be the common outcome of a variety of pathologies including infections, disruption of maternal–fetal tolerance and premature senescence . PTL may end in PT. However, in about 50% of cases birth occurs normally at term (≥37 weeks). These latter cases are referred to as SPL. While historically SPL has been considered neutral for posterior health, evidence is accumulating that risks are associated with SPL. Our results support the hypothesis that specific placental alterations are associated with PTL, independent of the time of birth. We predict that further research will underscore the relevance of PTL as a risk factor per se for placental health and short and long-term health risk of the fetus.

Recently, genome-wide technology has been applied to maternal or cord blood samples (and tissues) in order to study potential biomarkers and predictors of PT [23,24,44]. However, such information is not yet available for placenta, and even less in the context of PTL and PT. We suggest the importance of obtaining this information, to better understand placental pathologies. The potential epigenetic differences in the placenta related to PTL we describe here may stimulate future efforts toward defining the placental epigenome and transcriptome of PTL.

An increasing number of data support a significant impact of placental methylation on placental and fetal heath. Misexpression of imprinted genes in placenta has clearly hinted toward a correlation with abnormal birthweight of infants [50], which in turn has been associated (both as undergrowth and as overgrowth) with metabolic as well as cognitive disorders [51]. However, the direct relationship between altered methylation and future health impact is still largely unknown and should be further investigated.

Summary points

- Placental methylation at the *NCAM1*, *CFB* and *PLAGL1 loci* in preterm labor (PTL) did differ significantly from controls.
- Altered placental DNA methylation at the *NCAM1* and *VEGFA loci* in PTL is associated with inflammation.
- Alterations in placental DNA methylation of selected *loci* appear associated with PTL *per se*, and independent of gestational age at birth.
- In a small cohort, altered levels of both placental DNA methylation and mRNA expression of *PLAGL1* appeared associated with PTL.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/epi-2019-0346

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Data sharing statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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