Early- and late-onset pre-eclampsia and the DNA methylation of circadian clock and clock-controlled genes in placental and newborn tissues

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ABSTRACT

The placenta is important in providing a healthy environment for the fetus and plays a central role in the pathophysiology of pre-eclampsia (PE). foetal and placental developments are influenced by epigenetic programming. There is some evidence that PE is controlled to an altered circadian homeostasis. In a nested case–control study embedded in the Rotterdam Periconceptional Cohort, we obtained placental tissue, umbilical cord leukocytes (UCL), and human umbilical venous endothelial cells of 13 early-onset PE, 16 late-onset PE and 83 controls comprising 36 uncomplicated and 47 complicated pregnancies, i.e. 27 foetal growth restricted and 20 spontaneous preterm birth. To investigate the associations between PE and the epigenetics of circadian clock and clock controlled genes in placental and newborn tissues, genome-wide DNA methylation analysis was performed using the Illumina HumanMethylation450K BeadChip and a candidate-gene approach using AN-COVA was applied on 939 CpGs of 39 circadian clock and clock-controlled genes. DNA methylation significantly differed in early-onset PE compared with spontaneous preterm birth at 6 CpGs in placental tissue (3.73E-5 $\leq$ p $\leq$ 0.016) and at 21 CpGs in UCL (1.09E-5$\leq$ p $\leq$ 0.024). In early onset PE compared with foetal growth restriction 2 CpGs in placental tissue (p < 0.05) and 8 CpGs in uncomplicated controls (4.78E-5$\leq$ p $\leq$ 0.049) were significantly different. Moreover, significantly different DNA methylation in early-onset PE compared with uncomplicated controls was shown at 6 CpGs in placental tissue (1.36E-4$\leq$ p $\leq$ 0.045) and 11 CpGs in uncomplicated controls (2.52E-6$\leq$ p $\leq$ 0.009). No significant associations were shown with late-onset PE between study groups or tissues. The most differentially methylated CpGs showed hypomethylation in placental tissue and hypermethylation in uncomplicated controls. In conclusion, DNA methylation of circadian clock and clock-controlled genes demonstrated most differences in UCL of early-onset PE compared with spontaneous preterm birth. Implications of the tissue-specific variations in epigenetic programming for circadian performance and long-term health need further investigation.

INTRODUCTION

The placenta serves as the maternal– foetal interface during pregnancy and is one of the main determinants of pregnancy course and outcome. It also functions as an endocrine organ and plays a major role in maternal adaptation to pregnancy and foetal growth and development (124, 125). Pre-eclampsia (PE) is a multiorgan disorder in which impaired placental functioning due to excessive oxidative stress is involved (1). PE can affect the maternal liver, kidneys, brain and clotting system and is often complicated with foetal growth restriction (FGR) and preterm birth (PTB).

The internal body clock is of main importance in the regulation of the metabolic and hormonal states during reproduction and pregnancy (126-129). All tissues and cells, including the placenta, have a circadian clock, which is kept synchronous by the master clock (130, 131). The circadian clock imposes 24-hour rhythmicity of behavior, physiology, and metabolism. The mammalian circadian system is regulated by the circadian master clock, located in the suprachiasmatic nuclei and receiving light input from the retina to keep synchrony with the light–dark cycle. Evidence is accumulating that circadian misalignment significantly impacts pregnancy and health during the life course (132). Recent reports, studying women working night shifts (in services such as healthcare, military, protection and airway), show associations between disrupting the light–dark cycle during pregnancy and a higher risk of PTB, low birth weight, spontaneous abortion and subfertility (133-135). So far, however, only a few data are available on associations between alterations of the circadian rhythm and PE (136-139). In this regard of interest is the hormone melatonin, which is regulated by the circadian clock and synthesized by the placenta thereby protecting against excessive oxidative stress involved in the pathogenesis of PE. (129, 140, 141). Epigenetic mechanisms implicated in foetal and placental development can explain associations between PE and increased risks of features of noncommunicable diseases in mothers and children (14-16, 142). Altered DNA methylation patterns are found in placental tissues of PE and are associated with maternal blood pressure and excessive oxidative stress (143). Approximately 10% of the transcriptome is under circadian regulation, and, at the molecular level, the circadian oscillator is composed of a set of clock genes that act in interconnected transcription/translation feedback loops (144, 145) (see Figure 1 for a schematic overview of the regulation of the molecular clock). ARNTL (also known as BMAL1) and CLOCK are the positive activators and drive the transcription of clock genes by binding to E-box elements on their promoters. PER and CRY inhibit the specific transcription activation by ARNTL and CLOCK, thereby forming a negative feedback loop. An additional loop is formed by NR1D1 (also known as Rev-Erbα), which represses ARNTL transcription. The period length of the circadian clock cycle is fine-tuned by posttranslational modifications, such as phosphorylation and ubiquitylation, that regulate clock protein stability.

Figure 1. Circadian clock
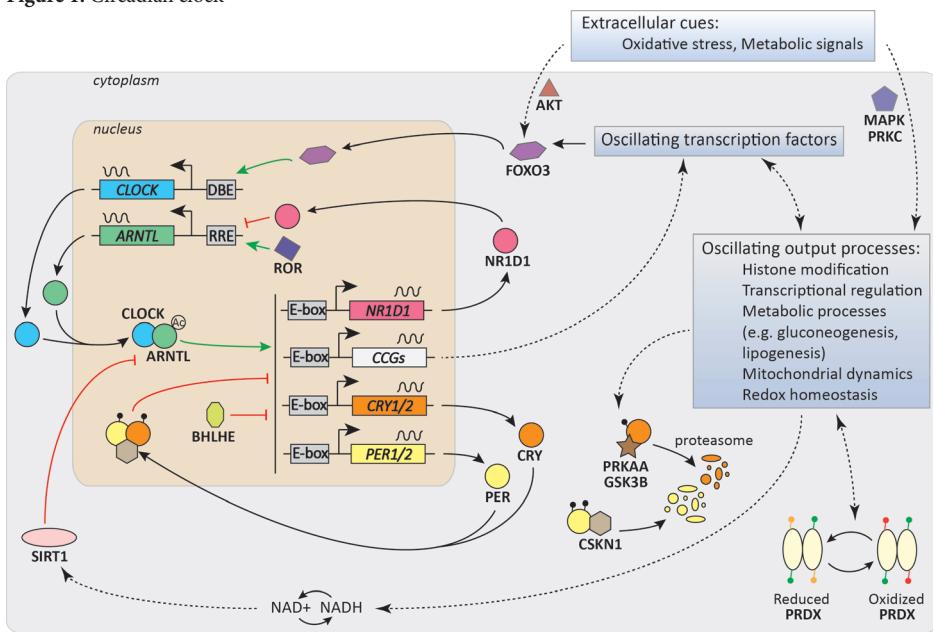
Model of the circadian core oscillator, showing the main core clock genes and transcription/translation feedback loops, as well as clock (controlled) genes (CCGs) that link the molecular clock to oscillating output processes. The core clock proteins are depicted as circles (CLOCK, ARNTL, CRY, PER, NR1D1).

From this background, we *hypothesize* that PE is associated with alterations in tissue-specific epigenetic programming of circadian clock (controlled) gene loci in placental and newborn tissues. The objective of our study was to examine the DNA methylation of circadian clock and clock-controlled genes in the placenta, umbilical cord leukocytes (UCL) and human umbilical vein endothelial cells (HUVEC) in relation to PE. Because of clear differences in pathophysiology, we selected a group of early-onset PE (EOPE, onset <34 weeks of gestation) and late-onset PE (LOPE, onset ≥34 weeks of gestation) (37, 146). In addition, because PE is often complicated with FGR and PTB, conditions known to also affect epigenetic programming, we investigated the DNA methylation of PE in comparison with non-PE controls, FGR and spontaneous PTB as additional non-PE control groups.

MATERIAL AND METHODS

Study design

A nested case-control study was conducted and embedded in the Rotterdam Periconceptional Cohort (Predict Study), a prospective tertiary hospital-based study of the Department of Obstetrics and Gynecology, Erasmus MC, University Medical Centre, Rotterdam,

the Netherlands (147). The study has been approved by the local Medical Ethical and Institutional Review Board of the Erasmus University Medical Centre (MEC-2004-227). At enrolment, all participants signed written informed consent before participation.

Patients with EOPE and LOPE were enrolled as well as controls without PE. Controls were divided in three different study groups: uncomplicated controls (CTRL), pregnancies complicated by FGR and pregnancies complicated by spontaneous PTB. The rational is that EOPE often results in PTB and FGR, and including these complicated pregnancy study groups provides the opportunity to compare EOPE with pregnancies of a comparable gestational age and birth weight.

PE was defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg on at least two occasions 4 hours apart after 20 weeks of gestation with proteinuria (protein/creatinine ratio of ≥30 mg/mmol). EOPE was defined as the onset of the disease <34 weeks of gestation (37, 146). FGR was defined as a birth weight below the 10th percentile. Patients were included based on the estimated foetal weight of ultrasound measurements during pregnancy. This was validated with a birth weight below the 10th percentile according to the reference curves of the Dutch Perinatal Registry. PTB was defined as delivery before 37 weeks of gestation. CTRL were pregnancies without PE, gestational hypertension, FGR or PTB. Clinical data were obtained from questionnaires and medical records.

Sample collection and DNA extraction

UCL: Umbilical cord blood was collected in anticoagulant vacutainer tubes (ethylenediaminetetraacetic acid) and processed within 48 hours after delivery to determine white blood cell count and differentiation and to store the isolated total white blood cell pellets at −80 degrees Celsius C until DNA extraction. Prior to DNA extraction, thawed UCL were subjected to erythrocyte lysis by use of Erythrocyte Lysis Buffer (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Placental tissue: After removal of the membranes and 2 mm of the top placental layer, placental biopsies (0.5 cm^3) were taken at four different sites of the foetal side of the placenta in a radius of 3 cm around the umbilical cord insertion. Samples were washed in phosphate buffered saline solution (PBS), frozen in liquid nitrogen and stored at −80 degrees Celsius C until DNA extraction. Placental tissue (30 mg) was grinded manually on dry ice. The obtained frozen powder was immediately added to lysis buffer and stored at −80 degrees Celsius C until further processing.

HUVEC: The umbilical cord vein was rinsed with cord buffer once and all umbilical cord blood was removed, filled with collagenase solution (1 mg/mL). After incubation for 15 min in a PBS water bath (37 degrees Celsius C), detached HUVEC were obtained and purified by magnetic-activated cell separation (MACS) with CD146 MACS MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). HUVEC pellets were frozen in liquid nitrogen and stored at −80 degrees Celsius C until DNA extraction.

Figure 2. Flowchart of the study population
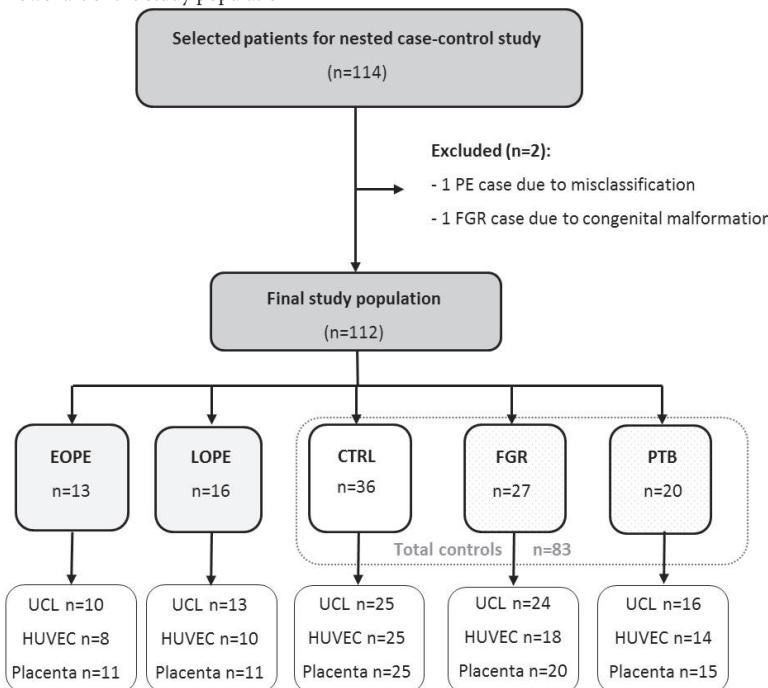
EOPE, early- onset pre-eclampsia; LOPE, late onset pre-eclampsia; CTRL, uncomplicated controls, FGR, foetal growth restriction, PTB, preterm birth; UCB, umbilical cord white blood cells; HUVEC, human umbilical vein endothelial cells

The collection and storage process was done in a standardized way by trained researchers.

Genomic DNA was extracted from all tissues using the Allprep DNA/RNA isolation mini kit (Qiagen, Hilden, Germany), using the manufacturer's protocol, and quantified using absorbance spectrometry.

DNA methylation measurement and pathway driven gene selection

Isolated DNA (500 ng) was treated with sodium bisulfite using the EZ-96 DNA methylation kit (Shallow Well Format) (Zymo Research, Irvine, CA). DNA methylation was measured by using Illumina HumanMethylation450K BeadChip. The methylation module of GenomeStudio (Illumina Inc., San Diego, CA) was used to check the performance of built-in quality controls in the Control Dashboard. CpGs located on the X-chromosome and CpGs containing known single nucleotide polymorphisms were excluded from analysis (148). Data were normalized by using the Dasen procedure. Methylation β-values were converted to M-values (M-value = $\log_2$ [β-value/(1-β-value)]). To achieve a pathway-driven analysis of DNA methylation at circadian clock (controlled) gene loci, a total of 39 genes were selected (Figure 3). This included core clock genes, clock-controlled genes, as well as output genes.

Considering that PE is associated with metabolic derangements, we especially focused on output genes that are involved in metabolic processes or that have been shown to have a link with the circadian clock and metabolic queues, such as insulin or glucose levels (150)(150) (147)(147)(147)(147)(147)(147)(147)(146) , (151, 152). As shown in Figure 3, for these 39 genes there are 939 CpGs present in the Illumina 450K array. These CpGs are distributed across the gene loci and are present in CpG-islands around the transcription start, at gene body and at regulatory regions such as the 5'- and 3'-UTR.

Figure 3. Selection of circadian clock and clock-controlled genes and CpGs.

Gene *	CpG (n)	Gene *	CpG (n)	Gene *	CpG (n)
AKT1	41	FOXO3	44	PRDX6	15
ARNTL	23	GSK3B	19	PRKAA1	9
ARNTL2	13	MAPK1	18	PRKAB1	11
BHLHE40	19	NPAS2	34	PRKACA	23
BHLHE41	16	NR1D1	24	PRKCA	79
CLOCK	14	NR1D2	17	RORA	106
CRTC1	40	PER1	18	RORB	11
CRY1	14	PER2	24	RORC	13
CRY2	24	PER3	25	SIRT1	17
CSNK1E	24	PRDX1	17	SIRT2	18
DBP	20	PRDX2	12	STRA13	22
FGF21	10	PRDX3	16	TIMELESS	15
FOXO1	33	PRDX5	22	WEE1	19

* The grey shaded gene names are core clock genes and the remaining gene names circadian clock related genes

Genes total: 39
CpG total: 939

AKT1: gene; V-Akt: murine thymoma viral oncogene homolog1; ARNTL: aryl hydrocarbon receptor nuclear translocator-like; ARNTL2: aryl hydrocarbon receptor nuclear translocator-like 2; BHLHE40: basic helix-loop-helix family, member e40; BHLHE41: basic helix-loop-helix family, member e41; CLOCK: clock circadian regulator; CRTC1: CREB-regulated transcription coactivator 1; CRY1: cryptochrome circadian clock 1; CRY2: cryptochrome circadian clock 2; CSNK1E: casein kinase 1, epsilon; DBP: D site of albumin promoter (albumin D-box) binding protein; FGF21: fibroblast growth factor 21; FOXO1: forkhead box O1; FOXO3: forkhead box O3; GSK3B: glycogen synthase kinase 3 beta; MAPK1: mitogen-activated protein kinase 1; NPAS2: neuronal PAS domain protein 2; NR1D1: nuclear receptor subfamily 1, group D, member 1; NR1D2: nuclear receptor subfamily 1, group D, member 2; PER1: period circadian clock 1; PER2: period circadian clock 2; PER3: period circadian clock 3; PRDX1: peroxiredoxin 1; PRDX2: peroxiredoxin 2; PRDX3: peroxiredoxin 3; PRDX5: peroxiredoxin 5; PRDX6: peroxiredoxin 6; PRKAA1: protein kinase, amp-activated, alpha 1 catalytic subunit; PRKAB1: protein kinase, AMP-activated, beta 1 non-catalytic subunit; PRKACA: protein kinase, CAMP-dependent, catalytic, alpha; PRKCA: protein kinase C, alpha; RORA: RAR-controlled orphan receptor A; RORB: RAR-controlled orphan receptor B; RORC: RAR-controlled orphan receptor C; SIRT1: sirtuin 1; SIRT2: sirtuin 2; STRA13: stimulated by retinoic acid 13; TIMELESS: timeless circadian clock; WEE1, WEE1 G2 checkpoint kinase.

Statistical analysis

For statistical analysis of clinical characteristics, analysis of variance (ANOVA) with post hoc Dunnett *t*-test for pairwise comparisons was used to compare continuous variables between experimental groups. Chi-square or Fischer's exact testing was used to compare categorical variables. For skewed data Kruskal–Wallis with post hoc Mann–Whitney tests were used.

To test for statistically differentially methylated CpGs (DMCs) in EOPE and LOPE compared to (un)complicated control groups, ANOVA and analysis of covariance (ANCOVA) with post hoc Bonferroni adjustment for multiple comparisons were performed. Although we included spontaneous PTB as an additional control group, in the ANCOVA model adjustments were made for gestational age at delivery and batch effect. Regarding UCL, adjustment for total number of UCL ($\times 10^9$/L) was performed. This information was available from flow-cytometric data of 61 samples (70%). Data imputation was used for missing data on leukocyte counts. We considered results to be statistically significant when $p < 0.05$. All statistical analyses were performed with R (R: A language and Environment for Statistical Computing, version 3.1.3, 2015 for Windows, R Core Team, Vienna, Austria).

RESULTS

Clinical characteristics

DNA methylation data were available of a total of 114 pregnancies. Exclusion of two pregnancies, which did not meet the inclusion criteria, resulted in a total sample of 112 pregnancies, consisting of 13 patients with EOPE, 16 patients with LOPE, 36 uncomplicated controls, 27 pregnancies complicated by FGR and 20 by spontaneous PTB. In Table 1, maternal and newborn characteristics are shown. In line with the selection criteria, blood pressure, gestational age and birth weight differed between PE and (un)complicated control groups. Patients with EOPE and LOPE were more often nulliparous compared to uncomplicated controls (respectively 84.6% and 81.3%, versus 30.6%, $p = 0.001$). In the EOPE group, more pregnancies were terminated by cesarean section compared to all other groups. Regarding the newborn characteristics, there were no significant differences in gender between the study groups. Gestational age and birth weight differed between all study groups. Although gestational age was still lower in EOPE compared to spontaneous PTB, these two groups were most comparable in gestational age.

Identification of DMCs between all study groups

As shown in Figure 4, we found significant differences in CpG methylation of clock (controlled) genes in all analyzed tissues. The number of DMCs was tissue-specific and most DMCs were found in UCL ($n = 31$), followed by placenta ($n = 7$) and HUVEC ($n = 1$).

Table 1. Maternal and new born characteristics

Maternal characteristics (n=112)	EOPE (n=13)	LOPE (n=16)	CTRL (n=36)	FGR (n=27)	PTB (n=20)
Age (years)	30.0 ± 4.7	33.3 ± 4.5	31.8 ± 5.1	29.7 ± 6.0	31.0 ± 4.8
Nulliparous, n (%)	11 (84.6)	13 (81.2)	11 (30.6)*#	17 (63.0)	10 (50.0)
Peak systolic BP (mmHg)	169.5 ± 24.2	151.4 ± 11.8	125.7 ± 12.2*#	129.9 ± 14.3*#	124.9 ± 14.6*#
Peak diastolic BP (mmHg)	98.2 ± 19.1	93.9 ± 11.2	74.8 ± 7.5*#	76.8 ± 9.9*#	76.6 ± 13.1*#
Caesarean section, n (%)	11 (84.6)	5 (31.3)*	10 (27.8)*	9* (33.3)	4 (20.0)*
Geographical origin, n (%)					
Western	12 (92.3)	9 (56.3)	30 (83.4)	17 (63.0)	18 (90.0)
Non-Western	1 (7.7)	7 (43.8)	6 (16.7)	10 (37.0)	2 (10.0)
Preconception BMI (kg/m	24.7 (10.1)	24.1 (4.4)	23.7 (4.8)	23.0 (6.5)	23.8 (6.1)
Smoking (yes), n (%)	2 (18.2)	0 (0.0)	0 (0.0)	2 (8.0)	2 (10.5)1
Co-morbidity (yes), n (%)	2 (15.4)	10 (62.5)	19 (52.8)	11 (40.7)	8 (40.0)
Newborn characteristics					
Male gender, n (%)	3 (23.1)	7 (43.8)	21 (58.3)	15 (55.6)	10 (50.0)
Gestational age at birth[a] (weeks)	30.7 (3.4)	37.4 (1.9)*	39.9 (1.9)*#	38.9 (2.6)*	35.4 (7.9)*#
Birth weight[a] (grams)	1185 (481)	3183 (1244)*	3713 (551)*#	2630 (595)*#	2660 (1805)*#
Birth weight <10th percentile, n (%)	1 (7.7)	3 (18.8)	0# (0.0)	27 (100.0)*#	0 (0.0)#

*Data represented as mean (± standard deviation) with corresponding ANOVA testing to examine overall differences between EOPE, LOPE, CTRL, FGR and PTB, followed by the post hoc Dunnett t-test for pairwise comparisons versus both EOPE and LOPE. Data represented as number (%) with corresponding Chi²/Fischer's exact testing. [a] Skewed data represented as median (interquartile range) with corresponding Kruskall-Wallis testing and post hoc Mann-Whitney testing. * p <0.05 versus EOPE pregnancies. # p <0.05 versus LOPE pregnancies. ANOVA analysis of variance; BP blood pressure; BMI body mass index; UCB umbilical cord blood; EOPE early onset pre-eclampsia; LOPE late onset pre-eclampsia; FGR foetal growth restriction; PTB preterm birth.*

Comparisons of EOPE and LOPE versus (un)complicated controls (Figure 5)

EOPE significantly differed from spontaneous PTB at six clock (controlled) CpGs in placental tissue, of the genes: *AKT1* ($p = 8.10^{E-5}$), *BHLHE41* ($p = 0.001$), *CSKN1E* ($p = 4.03^{E-5}$), *PRDX1* ($n = 2$, $p = 3.73^{E-5}$ and $p = 0.016$), and *RORA* ($p = 0.014$). As shown in Figure 6, these CpGs were hypomethylated in EOPE. In addition, in EOPE compared with spontaneous PTB, 21 CpGs were differentially methylated in UCL of the core clock genes: *ARNTL* ($p = 0.001$), *ARNTL2* ($p = 0.022$), *CLOCK* ($p = 1.40^{E-4}$), *CRY2* ($n = 2$, $p = 0.001$ and $p = 1.84^{E-4}$), *PER2* ($p = 7.72^{E-5}$), *NPAS2* ($n = 4$, $2.57^{E-4} < p < 0.035$); and clock-controlled genes: *CSKN1E* ($p = 0.003$), *FGF21* ($p = 4.15^{E-4}$), *FOXO1* ($p = 0.015$), *GSK3B* ($p = 0.024$), *MAPK1* ($p = 1.09^{E-5}$), *PRDX5* ($p = 0.003$), *PRKAA1* ($p = 0.002$), *PRKCA* ($n = 3$, $1.39^{E-5} < p < 3.89^{E-4}$) and *RORA* ($p = 0.018$). As shown in Figure 6, most CpGs were hypermethylated in EOPE.

In EOPE compared with FGR, we assessed two DMCs in placental tissue of the genes: *PER1* ($p = 0.041$) and *PRDX1* ($p = 0.001$) and eight DMCs were found in UCL in the same comparison of groups of the core clock genes: *CLOCK* ($p = 0.024$) and *PER2* ($p = 0.049$);

and clock-controlled genes: *CSKN1E* ($p = 0.007$), *FGF21* ($p = 0.017$), *FOXO3* ($p = 0.033$), *MAPK1* ($p = 4.78^{E-5}$), *PRDX5* ($p = 0.044$) and *PRKCA* (0.001).

EOPE differed from uncomplicated controls at six circadian clock (controlled) CpGs in placental tissue of the genes: *AKT1* ($p = 0.045$), *CRTC1* ($p = 0.020$), *CSKN1E* ($p = 0.017$), *PER1* ($p = 0.005$), *PRDX1* (1.36^{E-4}) and *RORA* ($p = 0.033$), and 11 DMCs were found in UCL in the same comparison of groups of the core clock genes: *ARNTL2* ($p = 0.009$), *CLOCK* ($p = 0.001$), *CRY2* ($n = 2$, $p = 0.009$) and *PER2* ($p = 0.007$), and clock-controlled genes: *FOXO3* (7.98^{E-5}), *MAPK1* ($n = 2$, $p = 0.006$ and $p = 2.52^{E-6}$), *PRDX5* ($p = 0.002$) and *PRKCA* ($n = 2$, $p = 0.006$ and $p = 0.005$).

While in the overall analysis one DMC was found in HUVEC, we found no DMCs in HUVEC when comparing EOPE and LOPE to all other study groups. This indicates that the DMCs identified with the overall analysis represent differences between control groups. Also no DMCs were found in LOPE. In Figure 6, absolute methylation levels are shown for the different groups at different gestational ages.

Figure 4. Differentially methylated CpGs for the three different tissues. An overall ANOVA was performed to test for differentially methylated CpGs ($p < 0.05$) between all study groups.

A   Placenta

Gene*	CpG name
AKT1	cg19831386
BHLHE40	cg02220837
BHLHE41	cg16047471
CSNK1E	cg01346718
PER1	cg10708955
PRDX1	cg09131901
PRDX1	cg07159951

B   UCL

Gene *	CpG name	Gene	CpG name
ARNTL	cg07032258	NPAS2	cg00562376
ARNTL	cg06499652	NPAS2	cg13371883
ARNTL2	cg26165146	NPAS2	cg20370783
CLOCK	cg04264638	PER2	cg23072629
CRY2	cg21002528	PRDX5	cg10718809
CRY2	cg24305156	PRKAA1	cg13345558
CSNK1E	cg14055351	PRKACA	cg03856723
CSNK1E	cg22494725	PRKCA	cg14648237
FGF21	cg21107581	PRKCA	cg15093766
FOXO1	cg10318492	PRKCA	cg15212295
FOXO1	cg24858665	PRKCA	cg16808922
FOXO3	cg04855107	PRKCA	cg23542572
FOXO3	cg13789303	PRKCA	cg26450228
MAPK1	cg05546044	RORA	cg13146553
MAPK1	cg18352793	RORA	cg20024192
		RORA	cg23411013

C   HUVEC

Gene	CpG name
MAPK1	cg12802921

* The grey shaded gene names are core clock genes and the remaining gene names circadian clock related genes

UCL: umbilical cord leukocytes; HUVEC: human umbilical vein endothelial cells

Figure 5. EOPE and LOPE compared to different control groups (ANCOVA). ANCOVA, adjusted for gestational age at delivery, batch effect, and leukocyte count for UCL only was performed. p Values are shown in the columns.

A Placenta

Gene *	CPG name	CPG type	EOPE vs. FGR	EOPE vs. PTB	EOPE vs. CTRL	LOPE vs. FGR	LOPE vs. PTB	LOPE vs. CTRL
AKT1	cg19831386	3' UTR	n.s.	8.10^{E-5}	0.045	n.s.	n.s.	n.s.
BHLHE41	cg16047471	3' UTR	n.s.	0.001	n.s.	n.s.	n.s.	n.s.
CRTC1	cg16623153	body	n.s.	n.s.	0.020	n.s.	n.s.	n.s.
CSKN1E	cg01346718	5' UTR	n.s.	4.03^{E-5}	0.017	n.s.	n.s.	n.s.
PER1	cg10708955	TSS1500	0.041	n.s.	0.005	n.s.	n.s.	n.s.
PRDX1	cg07159951	body	n.s.	0.016	n.s.	n.s.	n.s.	n.s.
PRDX1	cg09131901	body	0.001	3.73^{E-5}	1.36^{E-4}	n.s.	n.s.	n.s.
RORA	cg03707955	body	n.s.	0.014	n.s.	n.s.	n.s.	n.s.
RORA	cg24715928	body	n.s.	n.s.	0.033	n.s.	n.s.	n.s.

B UCL

Gene *	CPG name	CPG type	EOPE vs. FGR	EOPE vs. PTB	EOPE vs. CTRL	LOPE vs. FGR	LOPE vs. PTB	LOPE vs. CTRL
ARNTL	cg07032258	5' UTR	n.s.	0.001	n.s.	n.s.	n.s.	n.s.
ARNTL2	cg26165146	TSS1500	n.s.	0.022	0.009	n.s.	n.s.	n.s.
CLOCK	cg04264638	5' UTR	0.024	1.40^{E-4}	0.001	n.s.	n.s.	n.s.
CRY2	cg21002528	3' UTR	n.s.	0.001	0.009	n.s.	n.s.	n.s.
CRY2	cg24305156	body	n.s.	1.84^{E-4}	0.002	n.s.	n.s.	n.s.
CSKN1E	cg14055351	3' UTR	n.s.	0.003	n.s.	n.s.	n.s.	n.s.
CSKN1E	cg22494725	body	0.007	n.s.	n.s.	n.s.	n.s.	n.s.
FGF21	cg21107581	TSS1500	0.017	4.15^{E-4}	n.s.	n.s.	n.s.	n.s.
FOXO1	cg24858665	body	n.s.	0.015	n.s.	n.s.	n.s.	n.s.
FOXO3	cg04855107	body	0.033	n.s.	7.98^{E-5}	n.s.	n.s.	n.s.
GSK3B	cg12090885	body	v	0.024	n.s.	n.s.	n.s.	n.s.
MAPK1	cg05546044	TSS1500	n.s.	n.s.	0.006	n.s.	n.s.	n.s.
MAPK1	cg18352793	body	4.78^{E-5}	1.09^{E-7}	2.52^{E-6}	n.s.	n.s.	n.s.
NPAS2	cg00562376	body	n.s.	0.020	n.s.	n.s.	n.s.	n.s.
NPAS2	cg13371883	5' UTR	n.s.	0.008	n.s.	n.s.	n.s.	n.s.
NPAS2	cg20370783	5' UTR	n.s.	2.57^{E-4}	n.s.	n.s.	n.s.	n.s.
NPAS2	cg21609584	5' UTR	n.s.	0.035	n.s.	n.s.	n.s.	n.s.
PER2	cg23072629	5' UTR	0.049	7.72^{E-5}	0.007	n.s.	n.s.	n.s.
PRDX5	cg10718809	body	0.044	0.003	0.002	n.s.	n.s.	n.s.
PRKAA1	cg13345558	body	n.s.	0.002	n.s.	n.s.	n.s.	n.s.
PRKCA	cg16808922	body	n.s.	1.39^{E-5}	0.006	n.s.	n.s.	n.s.
PRKCA	cg23542572	body	0.001	4.53^{E-5}	0.005	n.s.	n.s.	n.s.
PRKCA	cg26450228	body	n.s.	3.89^{E-4}	n.s.	n.s.	n.s.	n.s.
RORA	cg23411013	body	n.s.	0.018	n.s.	n.s.	n.s.	n.s.

* The grey shaded gene names are core clock genes and the remaining gene names circadian clock related genes
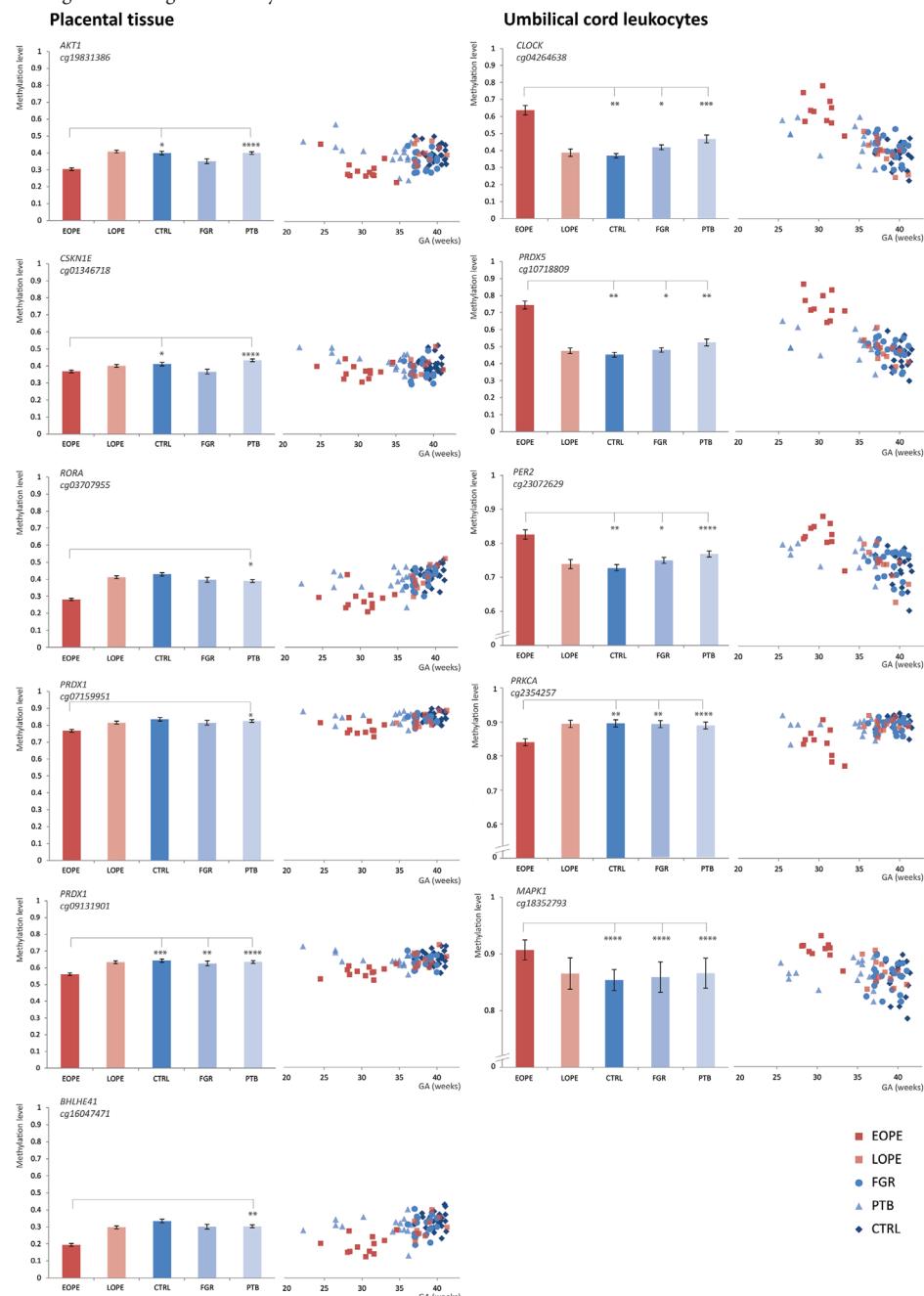

C Placenta
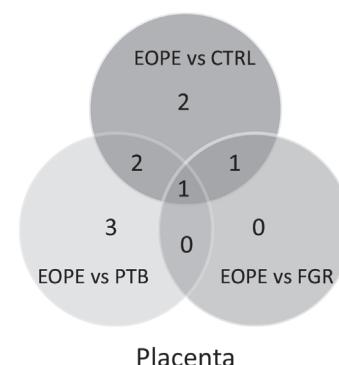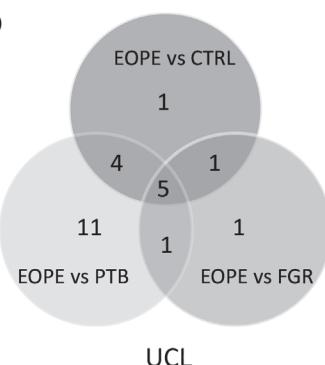
D UCL

EOPE: early-onset pre-eclampsia; LOPE: late-onset pre-eclampsia; CTRL: uncomplicated controls; FGR: foetal growth restriction; PTB: preterm birth; UCL: umbilical cord leukocytes; HUVEC: human umbilical vein endothelial cells; n.s.: not significant.

Figure 6. Absolute DNA methylation levels in the EOPE, LOPE and (un)complicated control groups depicted at the gestational age of delivery.

EOPE: early-onset pre-eclampsia; LOPE: late-onset pre-eclampsia; CTRL: uncomplicated controls; FGR: foetal growth restriction; PTB: preterm birth. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 1E-4, *****p < 1E-5.

DISCUSSION

We established tissue-specific alterations in methylation of circadian clock genes in EOPE compared to uncomplicated controls, FGR and spontaneous PTB, but not in any comparison with LOPE. In EOPE compared to spontaneous PTB, we identified in placental tissue DMCs in circadian clock-controlled genes, but not in the circadian core clock genes. In particular, the identification of DMCs in *AKT1* and *PRDX1* is in line with the oxidative stress imbalance in the placenta (Figure 1 shows the function of these genes). However, in UCL we identified DMCs in the circadian core clock genes *ARNTL, CLOCK* (and *NPAS2, a CLOCK homolog in some tissues*), *CRY2* and *PER2* in EOPE compared to spontaneous PTB. These genes are necessary for circadian clock function and changes in expression have been associated with noncommunicable diseases, such as metabolic disease and cancer (153, 154).

The most distinct differences were observed in EOPE compared to spontaneous PTB. Gestational age was most comparable between these two groups, which strengthens the hypothesis that PE is associated with differential methylation itself without confounding by gestational age. This is clearly demonstrated in the right part of Figure 6, where absolute DNA methylation levels in EOPE, LOPE and (un)complicated control groups are depicted at the gestational age of sampling. No differential methylation was observed between LOPE and all other (complicated) control groups. The epigenetic landscape is formed in particular during the periconceptional period and mainly during the first days after conception, supporting the evidence that LOPE largely develops after this period. The alterations in methylation as a memory of exposures in EOPE substantiate the first trimester origin of this PE phenotype. EOPE and LOPE are likely to reflect different underlying biological mechanisms, often indicated as the placental versus the metabolic syndrome (4).

Previous research showed that clock gene expression can be enhanced by hypoxia in human trophoblast cells *in vitro* (155), (135). Intermittent placental perfusion, caused by deficient trophoblast invasion of the endometrial arteries, can result in hypoxia-reoxygenation injury. The fluctuation in oxygen concentrations can cause excessive placental oxidative stress and as such alter the establishment of the epigenetic methylation markers (156). This is in line with our finding that in EOPE most DMCs were hypomethylated in placental tissue and hypermethylated in UCL and substantiated by other epigenetic studies of candidate genes in PE (157), (158-160), (161).

The opposite direction of methylation in placental tissue and UCL might be explained by differences in tissue sensitivity and the level of exposure. Although an important question is whether the observed changes in DNA methylation result in changes in gene expression, the current data cannot answer this question. Nevertheless, it is noteworthy that the same type of CpGs within a gene (i.e. promoter, body) show methylation changes in the same direction as the identified DMCs, although the differences are not significant (not shown).

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Although DNA methylation is considered to be mostly stable, there is a recent study showing that a number of CpGs show circadian oscillation in DNA methylation in mouse liver (162). In order to exclude a possible effect of the time of delivery, we performed an additional statistical analysis in which we did a sensitivity analysis for the time of delivery (supplemental figure, Figure S1). This did not affect the majority of the DMCs identified in the present study, indicating that time of delivery is not a confounding factor in our study.

Epigenetics and health of the offspring

In this study, we hypothesized that PE may be associated with changes in DNA methylation of circadian clock gene loci and leave epigenetic marks during pregnancy in newborn tissues. Alterations in DNA methylation of circadian clock and clock-controlled genes may be a consequence of a situation of excessive oxidative stress during exposure to this disorder. However, either cause or consequence, we showed in this study that an altered methylation level was present in some clock (controlled) genes in placental and foetal tissues after EOPE. This suggests that the epigenome of EOPE offspring might be affected as well. This is supported by our observation that differential methylation is higher in UCL than in placenta, suggesting that the alterations observed in placenta further induced epigenetic alterations in UCL (as a consequence of an imbalanced cellular environment due to placental malfunctioning and excessive oxidative stress). This is in line with the Developmental Origins of Health and Disease (DOHaD) paradigm (142). Unfortunately, we were not able to study DNA methylation of the offspring after follow-up. For future research, it is essential to validate these findings with data of the transcriptome and subsequently to measure DNA methylation and expression levels in these children and monitor the functioning of their circadian clock.

Strengths and limitations

As far as we know, this is the first study investigating DNA methylation of circadian clock-controlled genes in unique homogeneous study groups of EOPE and LOPE cases, and spontaneous PTB, FGR and uncomplicated controls. We were able to study three different tissues in most pregnancies collected in a standardized and high-quality manner. The external validity of the study is limited which is inherent to the tertiary hospital setting in which the study was performed. Moreover, the small number of samples, in particular of HUVEC, and the heterogeneity of cell types in placental tissues remain issues of potential bias (163, 164). Another limitation is that we do not have information about the circadian clock and light exposure of the participants to test for correlations with the results..

Conclusion

DNA methylation status of circadian clock and clock-controlled genes in placental tissue and UCL is different between patients with EOPE and non-PE controls. This may be explained

by a longer exposure to placental oxidative stress as compared to pregnancies complicated by LOPE. For future research, it would be interesting to study whether these DNA methylation alterations affect long-term health and circadian performance in the offspring.