



Early- and late-onset preeclampsia and the tissue-specific epigenome of the placenta and newborn



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ABSTRACT

Introduction: Preeclampsia (PE) carries increased risks of cardiovascular- and metabolic diseases in mothers and offspring during the life course. While the severe early-onset PE (EOPE) phenotype originates from impaired placentation in early pregnancy, late-onset PE (LOPE) is in particular associated with pre-existing maternal cardiovascular- and metabolic risk factors. We hypothesize that PE is associated with altered epigenetic programming of placental and fetal tissues and that these epigenetic changes might elucidate the increased cardiovascular- and metabolic disease susceptibility in PE offspring.

Methods: A nested case-control study was conducted in The Rotterdam Periconceptional Cohort comprising 13 EOPE, 16 LOPE, and three control groups of 36 uncomplicated pregnancies, 27 normotensive fetal growth restricted and 20 normotensive preterm birth (PTB) complicated pregnancies. Placental tissue, newborn umbilical cord white blood cells (UC-WBC) and umbilical vein endothelial cells were collected and DNA methylation of cytosine-guanine dinucleotides was measured by the Illumina HumanMethylation450K BeadChip. An epigenome-wide analysis was performed by using multiple linear regression models.

Results: Epigenome-wide tissue-specific analysis between EOPE and PTB controls revealed 5001 mostly hypermethylated differentially methylated positions (DMPs) in UC-WBC and 869 mostly hypomethylated DMPs in placental tissue, situated in or close to genes associated with cardiovascular-metabolic developmental pathways.

Discussion: This study shows differential methylation in UC-WBC and placental tissue in EOPE as compared to PTB, identifying DMPs that are associated with cardiovascular system pathways. Future studies should examine these loci and pathways in more detail to elucidate the associations between prenatal PE exposure and the cardiovascular disease risk in offspring.

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Abbreviations: PE, preeclampsia; EOPE, early-onset preeclampsia; LOPE, late-onset preeclampsia; UC-WBC, umbilical cord white blood cells; HUVEC, human umbilical vein endothelial cells; CpGs, cytosine-guanine dinucleotides; DMPs, differentially methylated positions; EWAS, epigenome-wide association studies; FGR, fetal growth restriction; PTB, preterm birth; OR, odds ratio; CI, confidence interval; GO-term, gene-ontology term; PBS, phosphate buffered saline-solution; MACS, magnetic activated cell separation; FDR, false discovery rate; bp, basepairs; IPA, Ingenuity pathway analysis; DAVID, Database for Annotation, Visualization and Integrated Discovery; PCA, Principal Component Analysis; non-CGI, non-CpG island.

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1. Introduction

Preeclampsia (PE) is a major cause of maternal and fetal morbidity and mortality and is suggested to be associated with the future risk of cardiovascular- and metabolic diseases in mothers and offspring [1]. Two main disease entities have been identified. Early-onset PE (EOPE) is the more severe phenotype originating from impaired placentation during the first trimester of pregnancy; late-onset PE (LOPE) occurs in the third trimester mainly as a consequence of exposure to pre-existing maternal cardiovascular- and metabolic risk factors [2,3]. The exact pathophysiology of PE is not understood, but it is known that in EOPE inadequate spiral artery remodelling leads to ischaemia-reperfusion-type placental insults [4]. This induces an excessive production of oxidative radicals. As a result, pro-inflammatory cytokines are released into the maternal circulation inducing endothelial dysfunction that leads to the clinical symptoms of PE [4]. Excessive placental oxidative stressors are also produced in LOPE but more towards the end of pregnancy [2].

PE is considered a complex disease induced by gene- and environmental interactions [5,6]. Altered epigenetic programming of specific tissues, induced by excessive oxidative stress, has been suggested to be an underlying mechanism [5]. Impaired placental perfusion, excessive production of oxidative radicals, endothelial dysfunction and immune modifications in PE may disturb epigenetic programming in offspring tissues as well, resulting in derangements of the vascular epigenome and function in the offspring [7–10]. From this background we *hypothesize* that altered epigenetic programming of especially placental tissues is associated with the pathogenesis of PE (as cause or consequence) and with cardiovascular and metabolic risks in the offspring over the life course [11].

Although PE has been associated with DNA methylation changes of candidate genes in placental and newborn tissue, only a few epigenome-wide association studies (EWAS) have found new loci of interest and most of these did not examine multiple tissues per patient [12–17]. In this study we examined tissue-specific genome-wide DNA methylation of umbilical cord white blood cells (UC-WBC), placental tissue and human umbilical cord endothelial cells (HUVEC) in relation to EOPE and LOPE.

Considering that PE is complicated by fetal growth restriction (FGR) in 12% of cases and associated with iatrogenic preterm birth (PTB) in 20%, which are conditions that can independently affect epigenetic programming, we examined the epigenome of PE and uncomplicated controls but also that of controls complicated by FGR and PTB [18–20].

2. Materials and methods

2.1. Study design

Pregnant women and their newborn babies were recruited between June 2011 and June 2013 in a nested case-control study embedded in The Rotterdam Periconceptional Cohort (Predict study), at the Erasmus MC, University Medical Centre Rotterdam, the Netherlands [21]. Cases comprised of EOPE and LOPE, and unmatched controls comprised of uncomplicated pregnancies and FGR and PTB complicated pregnancies. Patients were recruited for our case-control study by two different pathways. The first pathway contains subjects that were included in the Predict study in the first trimester and developed PE later in pregnancy. The second pathway contains subjects that were admitted to our hospital because of PE after the first trimester, and were specifically recruited in the Predict Study for our case-control study. This also applies to our (un)complicated control groups.

2.2. Maternal and fetal characteristics

PE was defined according to the International Society for the Study of Hypertension in Pregnancy as gestational hypertension of at least 140/90 mmHg accompanied by a urine protein/creatinine ratio of ≥ 30 mg/mmol arising de novo after the 20th week of gestation [22]. EOPE and LOPE were defined as being diagnosed before and after 34 weeks of gestation, respectively [23]. Uncomplicated pregnancies were defined as pregnancies without the following pregnancy-specific complications: PE, gestational hypertension, FGR or PTB. FGR was defined as an estimated fetal weight below the 10th percentile for gestational age based on ultrasound measurements performed between 20 and 38 weeks gestational age [24]. Birth weight percentiles were calculated using the reference curves of the Dutch Perinatal Registry to validate birth weight <10th percentile [25]. Spontaneous preterm deliveries between 22 and 37 weeks of gestation were defined as PTB [26]. Women with HIV infection, aged <18 years, not able to read and understand the Dutch language, multiple birth pregnancies or women with pregnancies complicated by fetal congenital malformations were excluded.

Maternal and newborn characteristics were obtained from hospital medical records. All women gave written informed consent before participation and parental informed consent was obtained for the child. The research has been carried out in accordance with the Declaration of Helsinki (2013) of the World Medical Association.

2.3. Data collection

UC-WBC samples from the umbilical vein were collected with the placenta still in situ. Thereafter, placental tissue was obtained and HUVEC were isolated and stored until DNA extraction. A detailed description of the data collection, UC-WBC processing, HUVEC isolation and DNA extraction is provided in [Appendix 1](#).

2.4. DNA methylation measurement

Isolated genomic DNA (500 ng) was treated with sodium bisulphite using the EZ-96 DNA methylation kit (Shallow) (Zymo Research, Irvine, CA, USA). Hybridization was performed following manufacturer's instructions. DNA methylation of CpGs was measured by the Illumina HumanMethylation450K BeadChip using the manufacturer's protocol (Illumina, Inc., San Diego, CA, USA) [27–29]. Data quality control and pre-processing is described in [Appendix 2](#).

2.5. Differentially methylated position (DMP) identification

To improve statistical power, prior to further statistical analysis a selection of probes containing at least ≥ 0.05 SD variability in methylation β -values across all samples was applied for each tissue separately [30]. This resulted in a remaining total of 43,488 UC-WBC probes, 134,700 placental probes and 42,352 HUVEC probes. Methylation β -values were converted to M-values using: $M\text{-value} = \log_2(\beta\text{-value}/(1-\beta\text{-value}))$ [31]. A multiple linear regression model was used predicting methylation M-values by disease state for EOPE and LOPE versus (un)complicated controls, adjusted for bisulphite-plate batch and gestational age as covariates for each tissue separately (R package 'CpGassoc'). Sensitivity analyses were performed for the following covariates: batch-effect of the bisulphite-plate, gestational age, birth weight, fetal gender, maternal comorbidity, mode of delivery and moment of inclusion for the study in or >1st trimester. Only gestational age and bisulphite-plate were thereafter included in our statistical model as

potential confounders.

UC-WBC analyses were performed with additional adjustment for total number of UC-WBC leucocytes ($\times 10^9/L$), available from our flow-cytometric data ($n = 61$, 70%) or otherwise imputed ($n = 27$, 30%) [32]. Missing leucocyte numbers were imputed by 'Multiple Imputations with Chained Equations' using other available UC-WBC variables and all contributing maternal and fetal characteristics with maternal age² as the best predictive variable (package mice) [33]. Additionally, Houseman-data was applied during imputation, further predicting proportions of lymphocytes, monocytes and granulocytes based on a community cohort of 94 non-diseased, non-smoking, predominantly female individuals between 24 and 45 years old, which improved the Akaike Information Criterion by 11 [34]. Imputed leucocyte concentrations are given in [Supplementary Table 1](#). Due to sample size constraints we could not adjust for further differentiated white blood cell counts.

We used the following model:

$$M_i = \beta_0 + \beta_1 G + \beta_2 GA + \beta_3 PLATE_2 + \beta_4 PLATE_3 + (+\beta_5 UC-WBC-leucocytes) + \varepsilon_i$$

where M_i is the (base 2) logit of the methylation of individual i . G is the group indicator variable (that is one if the sample corresponds to EOPE or LOPE and zero otherwise), GA is gestational age and $PLATE_2$ and $PLATE_3$ are indicator variables for the sample plates. β_5 is only applied for the UC-WBC analysis. ε_i is the residual error assumed to derive from a normal distribution.

A False Discovery Rate (FDR) adjusted p-value below 0.05 was considered significant. CpG sites were classified as DMP if statistically significant and presenting with a minimal effect-size ΔM of 0.8. We considered various M-value cut-off values for further data analysis and applied the widely used robust cut-off of 12.5% $\Delta\beta$

(~0.8 on M-value scale) ([Supplementary Table 2](#), [Supplementary Fig. 2](#)) [13,35]. For the comparison with the smallest number of samples ($n = 8$ HUVEC samples in EOPE), we were able to measure methylation differences of at least 10% $\Delta\beta$ or higher, with a power of 0.7 (sd of 0.05 and $FDR < 0.05$). An arbitrary selection of 100 epigenome-wide significant hits was subjected to a critical assessment of M-value plots to exclude potential outlier-driven hits or residual single nucleotide polymorphism-interference.

A detailed description of the DMP CpG-density- and gene-centric enrichment analysis, Gene Ontology (GO) term enrichment analysis and Ingenuity Pathway Analysis (IPA) is provided in [Appendix 3](#).

3. Results

All eligible pregnancies that met the inclusion criteria and provided sufficient tissues were included for the case-control analysis. After exclusion of 2 misclassified pregnancies the final study population consisted of 112 pregnancies. The 29 PE pregnancies comprised of 13 EOPE and 16 LOPE pregnancies, and controls included 36 uncomplicated and 27 FGR- and 20 PTB complicated control pregnancies ([Fig. 1](#)). The number of patients is a result of the initially aimed number of 25 samples per tissue per case-control group. Because we were unable to collect this exact amount of samples and because we obtained often more samples from one patient, the number of corresponding patients differs for each case-control group.

Maternal and newborn characteristics are shown in [Table 1](#). In addition to significantly different PE-specific parameters, such as blood pressure, proteinuria, gestational age and birth weight, nulliparity was more frequent in EOPE (84.6%) and LOPE (81.3%) than in uncomplicated controls (30.6%, overall $p = 0.001$). Caesarean

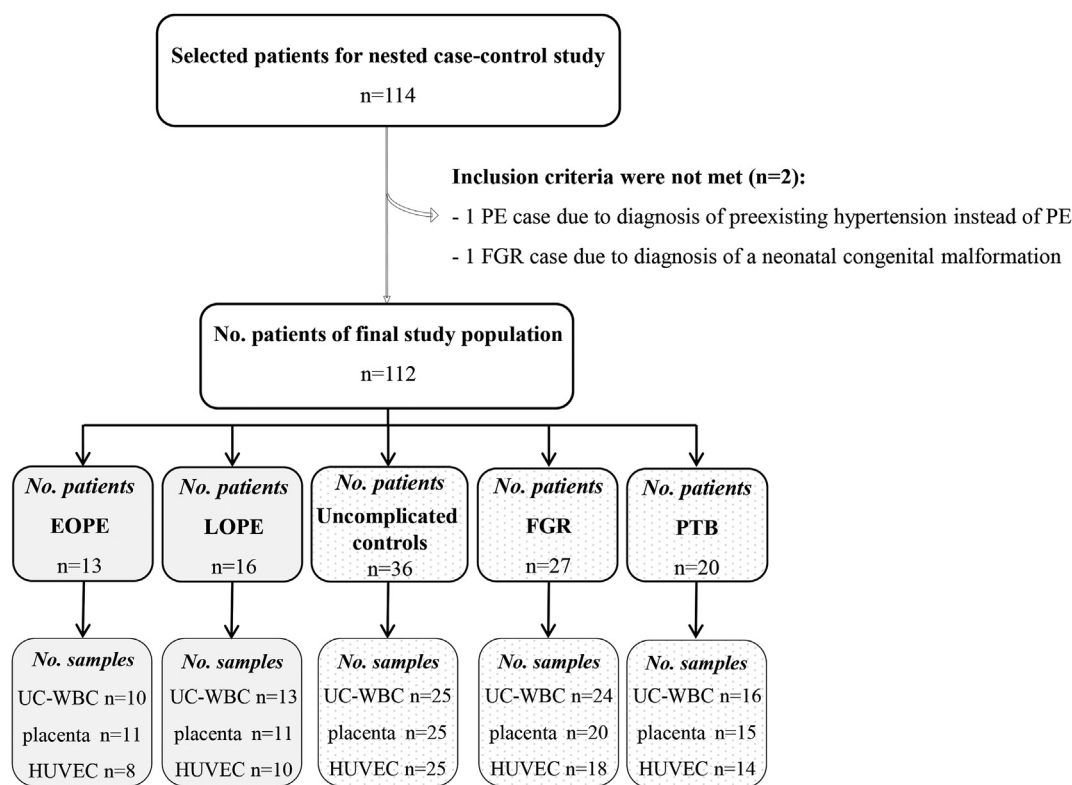


Fig. 1. Overview of the study population and sample distribution.

Legend: EOPE, early-onset preeclampsia; LOPE, late-onset preeclampsia; FGR, fetal growth restriction; PTB, preterm birth; UC-WBC, umbilical cord white blood cells; HUVEC, human umbilical vein endothelial cells; no., number of.

Table 1
Maternal and newborn characteristics.

Maternal characteristics	EOPE (n = 13)	LOPE (n = 16)	Uncomplicated controls (n = 36)	Complicated controls		Overall p-value
				normotensive FGR (n = 27)	normotensive PTB (n = 20)	
Age (years)	30.0 (4.7)	33.3 (4.5)	31.8 (5.1)	29.7 (6.0)	31.0 (5.1)	0.187
Nulliparous, n (%)	11 (84.6)	13 (81.3)	11*# (30.6)	17 (63.0)	10 (50.0)	0.001
Caesarean section, n (%)	11 (84.6)	5* (31.3)	10* (27.8)	9* (33.3)	4* (20.0)	0.002
Ethnicity, n (%)						
Western geographic origin	12 (92.3)	9 (56.3)	30 (83.4)	17 (63.0)	18 (90.0)	0.074
Non-Western geographic origin	1 (7.7)	7 (43.8)	6 (16.7)	10 (37.0)	2 (10.0)	
Preconception BMI ^a (kg/m ²)	24.7 (10.1)	24.1 (4.4)	23.7 (4.8)	23.0 (6.5)	23.8 (6.1)	0.668
Smoking during pregnancy (yes), n (%)	2 (18.2)	0 (0.0)	0 (0.0)	2 (8.0)	2 (10.5)	0.132
Comorbidity (yes), n (%)	1 (7.7)	7 (43.8)	8 (22.2)	7 (25.9)	4 (20.0)	0.260
Newborn characteristics						
Male gender, n (%)	3 (23.1)	7 (43.8)	21 (58.3)	15 (55.6)	10 (50.0)	0.253
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)	<0.001
Birth weight ^a (grams)	1185 (481)	3183* (1244)	3713*# (551)	2630*# (595)	2660*# (1805)	<0.001
Birth weight <10th percentile, n (%)	1 (7.7)	3 (18.8)	0# (0.0)	27*# (100.0)	0 (0.0)	<0.001

Data are presented as mean (standard deviation) with corresponding ANOVA testing to examine overall differences between the groups, followed by the post hoc Dunnett *t*-test for pairwise comparisons of EOPE and LOPE with (un)complicated controls.

Data are presented as number (%) with corresponding Chi2/Fischer's exact testing.

**p* < 0.05 versus EOPE pregnancies. #*p* < 0.05 versus LOPE pregnancies.

ANOVA, analysis of variance; BMI, body mass index; EOPE, early-onset preeclampsia; LOPE, late-onset preeclampsia; FGR, fetal growth restriction; PTB, preterm birth.

^a Non-parametric data are presented as median (interquartile range) with corresponding Kruskal-Wallis testing and posthoc Mann-Whitney testing.

section was more frequent in EOPE (84.6%) than in both LOPE (31.3%) and (un)complicated controls (20.0–33.3%, overall *p* = 0.002). In the EOPE group, one pregnancy was complicated by FGR (7.7%) and all by PTB; in the LOPE group, three pregnancies (18.8%) were complicated by FGR (*p* = 0.606, in comparison to the EOPE group) and three (18.8%) by PTB (*p* < 0.001, in comparison to the EOPE group).

3.1. DMP identification

Genome-wide DNA methylation data revealed an overall bimodal distribution for UC-WBC and HUVEC (Fig. 2). In these tissues, only a small amount of cytosine-guanine dinucleotides (CpGs) demonstrated methylation levels around 50%. In placental tissue however, more CpGs presented 50% methylation levels. Methylation levels in placental tissue and HUVEC were comparable in all

groups, but in EOPE the methylation levels in UC-WBC clearly deviated from those in all other groups.

A heatmap based on the clustering of methylation according to CpG and sample depicts prominent clustering of the three different tissues (Fig. 3A). We therefore also examined clustering per tissue, which demonstrated EOPE clustering in UC-WBC but no clustering in the placental- and HUVEC samples (Fig. 3B–D). This was validated by a principal component analysis (PCA) (Supplementary Fig. 1).

Differences between EOPE, LOPE and (un)complicated controls were examined in an epigenome-wide analysis. In the comparison of EOPE and PTB we found 12,040 (28%) differentially methylated CpGs in UC-WBC and 5,668 (0.5%) differentially methylated CpGs in placenta. One differentially methylated CpG each was found in the comparison of EOPE and uncomplicated controls in placental tissue and in HUVEC, and in the comparison of EOPE and FGR in placental

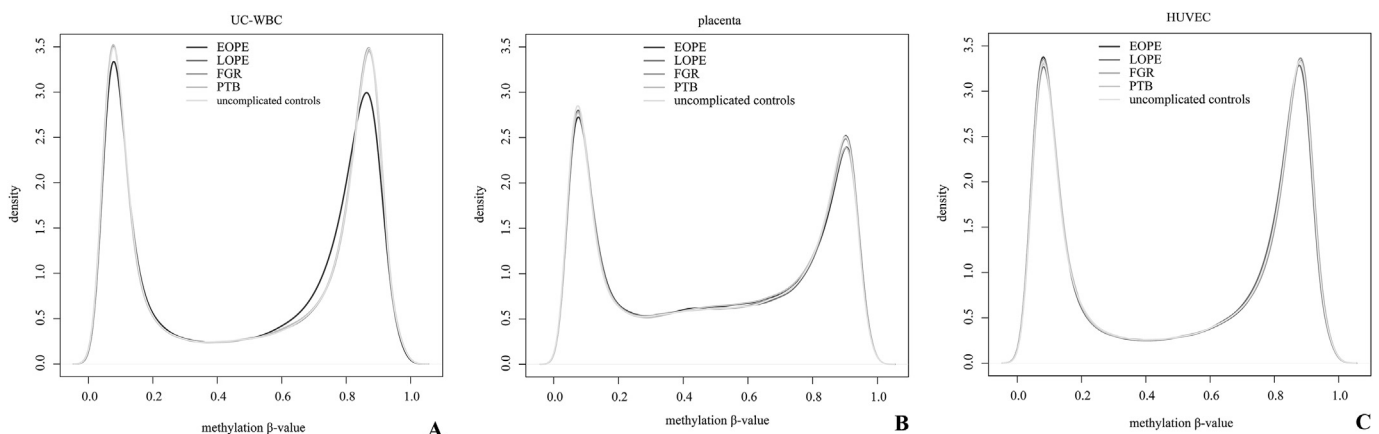


Fig. 2. Density plot of β -values per tissue and per group.

Legend: Density plots show the β -value-densities for UC-WBC (A), placenta (B) and HUVEC (C). Genome-wide DNA methylation data revealed an overall bimodal distribution of methylation levels. In addition, the placenta demonstrated a larger amount of CpGs with methylation levels around 50%, in contrast to the other tissues. In UC-WBC, EOPE deviates from LOPE and (un)complicated control pregnancies.

EOPE, early-onset preeclampsia; LOPE, late-onset preeclampsia; FGR, fetal growth restriction; PTB, preterm birth; UC-WBC, umbilical cord white blood cells; HUVEC, human umbilical vein endothelial cells; CpGs, cytosine-guanine dinucleotides.

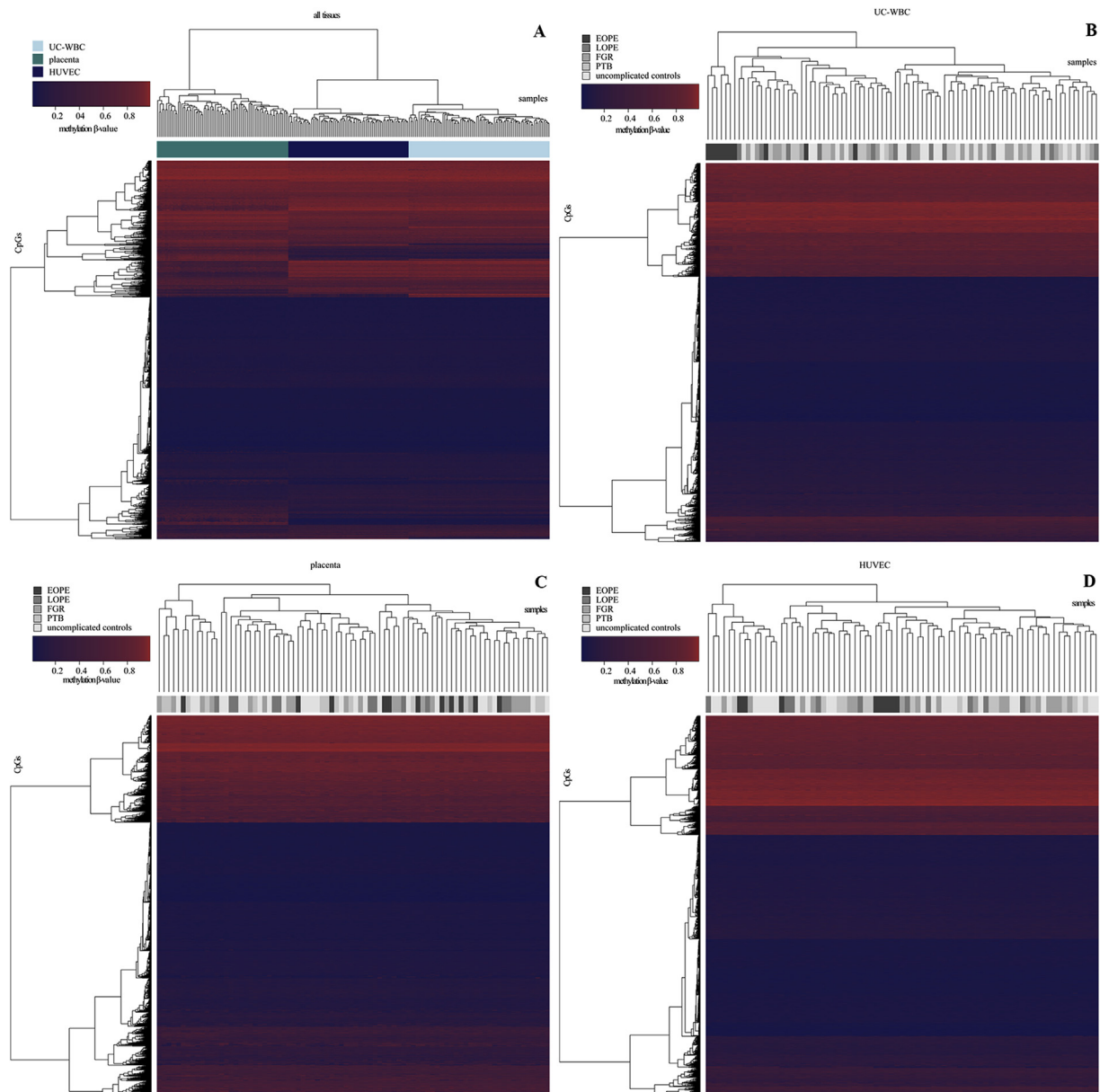


Fig. 3. Heatmap based on clustering of methylation (β -value) of all differentially methylated CpGs.

Legend: Heatmap plots show the clustering results of the EWAS using all significant CpGs in all tissues (A), UC-WBC samples (B), placental samples (C) and HUVEC samples (D). Samples are plotted on the horizontal axis and CpGs on the vertical axis. Samples cluster between the different tissues (A) and between EOPE and the (un)complicated control groups in UC-WBC samples (B). β -values are depicted for a better biological understanding of the figure.

EOPE, early-onset preeclampsia; LOPE, late-onset preeclampsia; FGR, fetal growth restriction; PTB, preterm birth; UC-WBC, umbilical cord white blood cells; HUVEC, human umbilical vein endothelial cells.

tissue (all FDR-adjusted $p < 0.05$). No epigenome-wide significant CpGs were found in the comparisons of LOPE and (un)complicated controls. Differentially methylated CpGs with an effect size >0.8 in M-value (logit2 of the β -value), representing an approximate change in β -value of 12.5%, were defined as differentially methylated positions (DMPs), which left 5,001 DMPs in UC-WBC and 869 DMPs in placenta in EOPE compared to PTB (Supplementary Table 2). 25 DMPs are overlapping between UC-WBC and placenta, which corresponds to 152 shared genes between UC-WBC and placenta. In UC-WBC, 71% of DMPs were hypermethylated; in contrast, hypomethylation was seen in 67% of the placental DMPs. Of the shared set of DMPs, 13 (52%) share the same methylation direction in both tissues and 54 (36%) genes are overlapping in

methylation direction (Fig. 4). All (overlapping) UC-WBC and placental DMP identifiers are listed in Supplementary Table 3.

3.2. CpG-density- and gene-centric enrichment of DMPs

To evaluate whether DMPs were enriched in certain genomic annotations, CpG island- and gene-centric annotations of UC-WBC- and placental DMPs were examined (Fig. 5 and Supplementary Table 4). DMP enrichment analysis relative to CpG islands revealed exactly opposite patterns for UC-WBC and placental DMPs with the strongest enrichment in non-CpG island regions and strongest depletion in CpG islands in UC-WBC (OR^{UC-WBC} 1.61, 95% CI 1.52–1.71 and OR^{UC-WBC} 0.23, 95% CI 0.20–0.26, respectively)

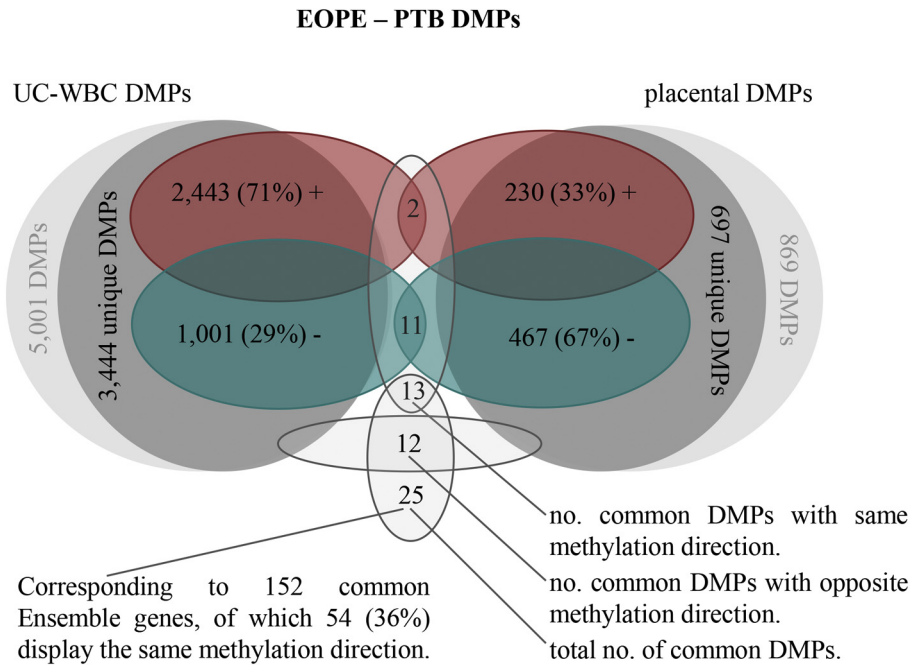


Fig. 4. Venn diagram of the number of (overlapping) UC-WBC- and placental DMPs in EOPE compared with PTB complicated pregnancies.

Legend: Red circles display the number of hypermethylated DMPs relative to PTB (+), green circles display the number of hypomethylated DMPs relative to PTB (-). UC-WBC, umbilical cord white blood cells; DMPs, differentially methylated positions; EOPE, early-onset preeclampsia; PTB, preterm birth; no., number of.

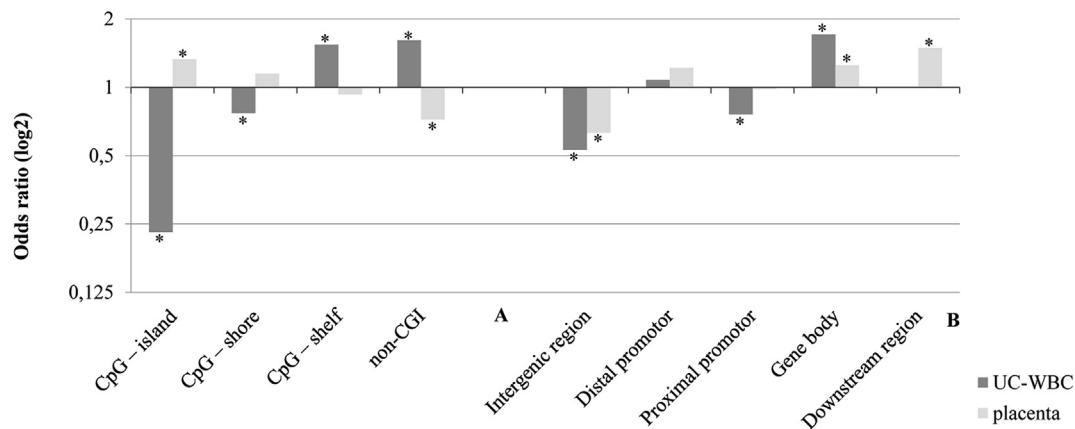


Fig. 5. UC-WBC- and placental- DMP enrichment in the CpG-density (A) and gene-centric (B) annotation, in EOPE compared to PTB complicated pregnancies.

Legend: **A.** DMP annotation relative to CpG islands showed exactly opposite patterns for UC-WBC as compared to placental DMPs with the strongest enrichment in non-CpG island regions and strongest depletion in CpG islands in UC-WBC versus strongest enrichment in CpG island regions and strongest depletion in non-CpG islands in placenta.

B. DMP annotation relative to genes, revealed strongest enrichment in gene body regions, with additional high enrichment in downstream regions of the placenta. A strong depletion of DMPs in intergenic regions was observed in both UC-WBC and placental tissue.

* $p < 0.05$. UC-WBC, umbilical cord white blood cells; EOPE, early-onset preeclampsia; PTB, preterm birth; non-CGI, non-CpG island; DMP, differentially methylated position.

versus strongest enrichment in CpG island regions and strongest depletion in non-CpG islands in placenta ($OR^{Placenta}$ 1.33, 95% CI 1.15–1.54 and OR^{UC-WBC} 0.72, 95% CI 0.62–0.83, respectively) (Fig. 5 and Supplementary Table 4).

The location of DMPs relative to genes revealed the strongest enrichment in gene bodies with additional high enrichment in downstream regions of the placenta. A strong depletion of DMPs in intergenic regions was observed in both UC-WBC and placental tissue (OR^{UC-WBC} 0.53, 95% CI 0.49–0.58, $OR^{Placenta}$ 0.63, 95% CI 0.53–0.75).

3.3. Gene-ontology (GO) term enrichment of DMP genes

To gain more insight in functionality, the DMPs were mapped to the nearest gene. This yielded 3,444 unique UC-WBC genes and 697 unique placental genes. The 3,444 UC-WBC DMP genes mapped to 2,744 genes in The Database for Annotation, Visualization and Integrated Discovery (DAVID) after discarding the remaining DMP annotations as transcripts. These genes were enriched for involvement in cell signalling processes and regulation of cell death, gene expression, RAS-activity, inflammatory responses and erythrocyte development. After excluding those DMPs that were mapped to transcripts instead of genes, DAVID mapped 536

placental DMP genes and revealed enriched GO term involvement in embryogenesis, regulation of transcription and cellular differentiation. UC-WBC and placental highest enriched GO terms are presented in Table 2.

3.4. Ingenuity pathway analysis (IPA) of DMP genes

We applied IPA to validate the GO findings from DAVID. Ingenuity software mapped 3,011 UC-WBC DMP genes and a canonical pathway analysis identified enrichment of top-ranked pathways all involved in cascade-signalling mechanisms. We further conducted network analyses, which revealed that the top networks were mainly associated with cardiovascular system development, cellular function and development, haematological system development and infectious diseases.

596 placental DMP genes were mapped by Ingenuity, of which enriched canonical pathways did not reach statistical significance. The top ten enriched networks were mainly associated with embryonic development, cellular development, regulation of gene expression, cardiovascular system development, cell death and survival and haematological system development. The highest enriched pathways and networks are presented in Table 3.

4. Discussion

In this study we demonstrate significant differences in genome-

Table 2
Top 10 DAVID GO Functional annotation clusters of EOPE UC-WBC- and placental DMP nearest genes.

UC-WBC	Enrichment score
1. Phosphorylation	4.6
2. Programming of cell death	3.9
3. Cytoskeleton organization	3.9
4. Positive regulation of gene expression	3.6
5. Regulation of RAS (GTPase) activity	3.3
6. Endocytosis	2.8
7. Regulation of phosphate metabolic process	2.7
8. Defence/inflammatory response	2.4
9. Erythrocyte differentiation and development	2.1
10. Regulation of endocytosis and phagocytosis	2.0
Placenta	Enrichment score
1. Embryonic development	7.3
2. Regulation of transcription	7.2
3. Neuron development	6.3
4. Embryonic morphogenesis	5.7
5. Positive regulation of transcription	4.9
6. Negative regulation of transcription	4.2
7. Embryonic morphogenesis	4.1
8. Respiratory development	3.9
9. Regulation of cell differentiation and development	3.5
10. Embryonic development: dorsal/ventral pattern formation	3.4

UC-WBC- and placental DMPs in EOPE were mapped to the nearest gene using Ensembl annotations from UCSC database. Assigned Ensembl genes were uploaded to the DAVID tool to examine possible enrichment of corresponding GO terms using the GO_BP_FAT annotation category. Fisher Exact was applied to measure the gene-enrichment of annotated GO terms of the uploaded gene list, against the whole human genome list as a background. To focus on the biology of the annotated GO terms, clusters of similar annotations were examined from the DAVID Functional annotation Clustering table tool. The clustering algorithm is based on the hypothesis that similar annotations have similar gene members, resulting in a Group Enrichment Score to rank their biological significance, which is based on the Fisher Exact p-values of each GO term within the cluster. The higher the enrichment score, the more enriched.

DMPs, differentially methylated positions; DAVID, Database for Annotation, Visualization and Integrated Discovery; GO-term, gene-ontology term; HUVEC, human umbilical vein endothelial cells.

wide UC-WBC and placental DNA methylation between EOPE and PTB controls but not between EOPE and FGR controls or uncomplicated controls. Significant differences were also not found between LOPE and all other (un)complicated control pregnancies.

4.1. EOPE differential methylation in comparison to PTB, in UC-WBC and placental tissue

Our most evident observation was the large difference in the level of differential methylation observed in EOPE but not in LOPE. This may be best explained by the excessive oxidative stress exposure in EOPE and LOPE pregnancies, suggested to be highly involved in the disturbance of epigenetic programming [7]. In response to various forms of stress, cells increase the production of important protective proteins while reducing the translation of other less relevant proteins to restore cellular homeostasis [36]. We observed both hyper- and hypomethylation in association with EOPE in UC-WBC and placenta tissue, and enrichment of both positive and negative regulation of transcription among the functional annotations of DMP genes, in line with these cellular stress responses. Moreover, if cellular stress responses fail or a sudden excessive stress event occurs, cellular death might occur, through apoptosis or necrosis [37]. Also this cellular death- and survival pathway was identified among the DMP gene functional annotations, underlining the potential involvement of excessive oxidative stress.

From our data it appears that oxidative stress may have more consequences for EOPE pregnancies, where placental and newborn tissues are indeed subjected to this adverse exposure from early pregnancy onwards. In EOPE, chorionic villi are exposed to oxidative stress at 8–9 weeks of gestation through contact with maternal oxygenated blood due to early unplugging of spiral arteries, disturbing the formation of the definitive placenta by restricted invasion and impaired remodelling of spiral arteries [36]. One might hypothesize that the epigenetic programming involved in this initial placental formation and differentiation is in fact already affected, perhaps inducing the further development of EOPE, rather than being a consequence of the disease. We suggest that perhaps the very initial process of epigenetic reprogramming between fertilisation and formation of the blastocyst is already affected in EOPE, explaining the observed extensive involvement of placental DMPs and also suggesting a more causative epigenetic involvement. In general, impaired placentation seems to play a much larger role in the pathophysiology of EOPE than LOPE, with more adverse consequences for the fetus, such as FGR, supporting the current results [2]. Also the maternal and fetal pro-inflammatory milieu is more pronounced in EOPE than in LOPE, with more potential consequences for UC-WBC epigenetic programming, as substantiated by our data [7].

Although we would have expected to establish some degree of differential methylation in LOPE, the lack of differential methylation is in accordance to previous literature [12,13,38–40]. Only Zhu et al. identified DMRs in placental tissue of severe LOPE pregnancies as compared to that of gestational age- and parity-matched controls. They however used different techniques (DNA immunoprecipitation and deep sequencing) and included only severe cases of LOPE, potentially explaining the discrepancy with our findings [16].

A possible reason for observing associations between EOPE and PTB only, is that these two groups are least affected by a larger gestational age range and additional comorbidity and hence easier to compare than the other control groups. The lack of any significantly differential methylation in HUVEC obtained from EOPE pregnancies might be partly explained by the decreased power as a result of the lower HUVEC sample size ($n = 8$) and the larger

Table 3

Top 10 Ingenuity networks and pathways of EOPE UC-WBC- and placental DMP nearest genes.

UC-WBC Canonical pathways	-log p-value
1. Molecular Mechanisms of Cancer	4,49
2. Tec Kinase Signalling	4,37
3. Relaxin Signalling	4,37
4. IL-1 Signalling	4,00
5. Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	3,87
6. PPAR Signalling	3,78
7. Renal Cell Carcinoma Signalling	3,74
8. G Beta Gamma Signalling	3,67
9. G-Protein Coupled Receptor Signalling	3,67
10. HGF Signalling	3,49
UC-WBC networks	Score
1. Cardiovascular System Development and Function, Lymphoid Tissue Structure and Development, Cell Morphology	29/35
2. Cardiovascular System Development and Function, Tissue Morphology, Cellular Movement	26/34
3. Cell Cycle, Cellular Development, Haematological System Development and Function	24/33
4. Connective Tissue Disorders, Developmental Disorder, Skeletal and Muscular Disorders	24/33
5. Cancer, Organismal Injury and Abnormalities, RNA Post-Transcriptional Modification	24/33
6. Cardiovascular Disease, Cellular Compromise, Organismal Injury and Abnormalities	23/32
7. Infectious Diseases, Antigen Presentation, Protein Synthesis	23/32
8. RNA Damage and Repair, Post-Translational Modification, Cellular Development	23/32
9. Cell Morphology, Cellular Function and Maintenance, Infectious Diseases	23/32
10. Cancer, Organismal Injury and Abnormalities, Reproductive System Disease	23/32
placental networks	Score
1. Embryonic Development, Hereditary Disorder, Neurological Disease	45/32
2. Cellular Development, Cellular Growth and Proliferation, Organismal Development	36/28
3. Gene Expression, Cancer, Organismal Injury and Abnormalities	36/28
4. Cell Cycle, Cellular Movement, Developmental Disorder	30/25
5. Cardiovascular System Development and Function, Cellular Movement, Carbohydrate Metabolism	25/22
6. Molecular Transport, Inflammatory Response, Haematological System Development and Function	14/15
7. Metabolic Disease, Organismal Injury and Abnormalities, Respiratory Disease	12/14
8. Cellular Development, Cellular Growth and Proliferation, Cellular Movement	12/14
9. Cell Death and Survival, Cell Cycle, Cellular Assembly and Organization	12/14
10. Cell Death and Survival, Cardiovascular System Development and Function, Cell Morphology	12/14

Ingenuity pathway analysis was performed with the annotated UC-WBC- and placental DMP Ensembl gene lists in EOPE with focus on canonical pathways and networks. Enriched canonical pathways were calculated using the Fisher's Exact test and subjected to Benjamini-Hochberg procedure for controlling FDR ($p < 0.05$). Networks were generated based on network eligible molecules, which were encoded by our DMP genes and also interact with other molecules in the Ingenuity Pathways Knowledge Base. A high score for a network indicates a more approximate fit between network eligible molecules and the molecules that constitute the network, calculated using the right-tailed Fisher's Exact Test. No significant EOPE placental canonical pathways were observed. DMPs, differentially methylated positions; HUVEC, human umbilical vein endothelial cells; FDR, false discovery rate.

variation, compared to (un)complicated controls.

The majority of DMPs in placental tissue was hypomethylated, which is substantiated by literature [13,38–41], whereas the more prominent hypermethylation in UC-WBC is in contrast to previous studies [14,42]. These studies did not adjust for potential confounders such as gestational age or leucocyte-count, which may explain the discrepancy of the results. Compared to the total number of DMPs, the relatively small shared set of 25 DMPs and 152 corresponding genes suggests that UC-WBC and placenta are behaving rather differently in association to EOPE. This is substantiated by the fact that only 52% of the shared DMPs and 36% of the shared genes in UC-WBC and placental tissue are behaving in the same methylation direction relative to PTB, and by the opposite enrichment patterns described below. In certain overlapping genes it appears that different CpGs are affected in UC-WBC than in placental tissue.

4.2. CpG-density- and gene-centric enrichment of EOPE UC-WBC- and placental DMPs

Enrichment analysis of the identified EOPE DMPs showed opposite patterns for the positions of UC-WBC- and placental DMPs relative to CpG-islands. EOPE was associated with strong differential methylation of non-CpG island regions in UC-WBC and depletion in CpG islands, whereas in placental tissue differential methylation was observed in CpG islands and depletion in non-CpG islands, which is in line with previous studies [13,14]. Evidence

emerges that CpG island methylation is mostly associated with long-term repression of gene expression such as imprinting, which is an important process for normal placental development [11,43]. Indeed, a supporting role of imprinting in the development of PE has been observed in previous studies [44].

UC-WBC- and placental DMPs display strong enrichment in gene bodies and evident depletion in intergenic regions. Previous studies describe enrichment of EOPE DMPs in placental enhancer- and UC-WBC 'open sea'-regions, classified according to the Illumina gene-centric annotation which is not directly comparable to our UCSC gene-centric annotation results. Gene body regions seem to be more susceptible to variation in DNA methylation, and may therefore represent interesting genomic regions for future epide-miologic epigenetic association studies [43]. Because methylation of gene bodies is generally associated with stimulation of trans-cription, the strong placental hypomethylation suggests mainly repression of transcription and the contrary for UC-WBC [43].

4.3. GO-term enrichment and IPA of EOPE DMP genes

DMP GO functional annotations demonstrated that genes linked to UC-WBC DMPs were involved in many processes associated with the pathogenesis of PE, such as regulation of inflammatory responses and RAS-activity [3,45]. We and others have previously described decreased leucocytes and increased nucleated red blood cells in EOPE UC-WBC, indeed suggesting inflammatory response involvement and disturbed fetal haematopoiesis [46,47]. Therefore,

of particular interest is the enriched GO term of erythrocyte development. The observed enriched GO-term of cellular death regulation might be related to flawing of the endothelial vessel wall in PE, causing apoptosis of endothelial cells, which has been related to the increased presence of endothelial microparticles in PE [7]. One of the most enriched UC-WBC GO terms covers a broad spectrum of cell signalling processes involved in many general processes, which is likely a result of general excessive oxidative stressors. The latter was confirmed by all highest significant canonical pathways from IPA. Placental DMP GO functions covered general functions such as cellular differentiation and cell death, embryogenesis and regulation of gene expression, which is in agreement to the impaired placentation and compromised fetal growth in EOPE and substantiated by literature [13].

The involvement of DNA methylation differences in cardiovascular programming of PE offspring was strongly suggested by many identified gene-ontology (GO) functional annotations and pathways associated with the DMP genes. Repeated enrichment of IPA genetic networks associated with development of cardiovascular and metabolic disease was observed. This is partly substantiated by previous PE EWAS, describing differential methylation in the fatty acid synthase pathway and in collagen-related genes [12,14]. There exists partial overlap between identified genes in association with EOPE in previous EWAS in both UC-WBC and placental DMP-genes, as depicted in [Supplementary Table 3](#). We believe these overlapping genes might represent interesting loci for further research and suggest a certain degree of reproducibility of our results. Yuen et al. identified hypomethylation of the *TIMP3* gene in EOPE placentas, suggesting reduced angiogenesis and trophoblast invasiveness by use of the Illumina GoldenGate Methylation assay [40]. In other vascular-related candidate gene studies, altered DNA methylation in association with PE was found of *LEP*, *VEGF*, *FLT-1*, *KDR*-genes and collagen-metabolism genes [39,41,48], substantiating the role of disturbed vascular programming in PE. All of these previously identified candidate genes were also present among our DMPs in UC-WBC and/or placental tissue.

Several studies have demonstrated that epigenetic programming may indeed be involved in the development of cardiovascular disease, as reviewed by Casanello et al. [49]. One of these studies showed increased DNA methylation of estrogen receptor beta promoters in endothelial cells of atherosclerotic plaques [50]. Alexander et al. showed that in smooth muscle cells epigenetic mechanisms were involved in the phenotypic switch to less contractility with vascular dysfunction as a result [51].

4.4. Strengths and limitations

The design of our EWAS is unique in that it addresses tissue-specific methylation levels of EOPE and LOPE and uses both uncomplicated and FGR- and PTB-complicated normotensive control pregnancies. The different tissues were obtained mostly from the same pregnancies and therefore the data provide novel insights in the tissue-specific epigenome-wide DNA-methylation in PE-exposed newborn tissues. Moreover, we adjusted for gestational age and UC-WBC cell mixtures [20,40], which has not often been done in previous studies [12,14,15]. The UC-WBC cell populations were however partly estimated, based on our own and external populations. Our description of the standardised tissue sampling is more extensive than in most other studies, and this is relevant because the method of tissue sampling can be an important potential confounder or effect modifier of DNA methylation [52,53].

Inherent to the observational and case-control character of the study, residual confounding cannot be fully excluded. The addition of birthweight as potential confounder did not improve our statistical model and was discarded also because of the limited sample

size. Moreover, we had no expression data to validate our findings and the external validity is reduced by the relatively high presence of comorbidities in uncomplicated controls in our tertiary hospital setting. Our findings could have improved with the use of larger sample sizes and homogeneous cell types of placental tissue and UC-WBC, thereby avoiding potential confounding by cell mixture variation, [54,55].

4.5. Conclusion

We found a large number of genome-wide DMPs in UC-WBC and placental tissue that were associated with EOPE. The enrichment in several cardiovascular system developmental pathways implies that epigenetic programming may be an underlying mechanism explaining the association with increased cardiovascular disease risks in EOPE offspring. Future follow-up studies in offspring should address whether these pathways represent potential targets for intervention or prevention of the enhanced cardiovascular disease susceptibility.

Contribution to authorship

EH contributed to the study design, data collection, laboratory work, statistical analysis and writing of the first draft and all revisions of the manuscript. AE contributed to the study design and writing of the manuscript. SW supervised and performed the statistical analysis. RS, KW, JF and JC contributed to the design of the statistical analysis and writing of the manuscript. AS and PS contributed to the bioinformatic analysis and writing of the manuscript. JM supervised the laboratory data analysis and contributed to the writing of the manuscript. RS initiated the study and supervised all aspects of the study and contributed to all versions of the manuscript. All authors contributed to the writing and revision of the manuscript and approved the final version.

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Disclosure of interest

None.

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Details of ethics approval

Ethical approval for the study was given by the Erasmus MC, University Medical Centre Research Ethics Board (MEC-2004-227).

Data availability

The datasets generated in the current study are available in the GEO repository with accession number GSE103253.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.placenta.2017.08.070>.

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