Early Onset Pre-Eclampsia Is Associated with Altered DNA Methylation of Cortisol-Signalling and Steroidogenic Genes in the Placenta

Kirsten Hogg1,4, John D. Blair1,4, Deborah E. McFadden2,4, Peter von Dadelzen3,4, Wendy P. Robinson1,4*

1 Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada, 2 Department of Pathology, University of British Columbia, Vancouver, British Columbia, Canada, 3 Department of Obstetrics and Gynaecology, University of British Columbia, Vancouver, British Columbia, Canada, 4 Child & Family Research Institute, Vancouver, British Columbia, Canada

Abstract

Placental cortisol is inactivated in normotensive pregnancies, but is frequently present in pre-eclampsia associated placenta. Since glucocorticoids are strongly associated with the programming of long-term health, we assessed DNA methylation of genes involved in cortisol signalling and bioavailability, and hormonal signalling in the placenta of normotensive and hypertensive pregnancies. Candidate genes/CpG sites were selected through analysis of Illumina HumanMethylation450 BeadChip array data on control (n = 19) and early onset pre-eclampsia (EOPET; n = 19) placental samples. DNA methylation was further quantified by bisulfite pyrosequencing in a larger cohort of control (n = 111) cases, in addition to EOPET (n = 19), late onset pre-eclampsia (LOPET; n = 18) and normotensive intrauterine growth restriction (nIUGR; n = 13) cases. DNA methylation (percentage points) was increased at CpG sites within genes encoding the glucocortocoid receptor (NR3C1 exon 1D promoter; +8.46%; P < 0.001) and corticotropin releasing hormone (CRH) binding protein (CRHBP intron 3; +9.14%; P < 0.05), and decreased within CRH (5’UTR: −4.30%; P < 0.05) in EOPET-associated placentae, but not in LOPET nor nIUGR cases, compared to controls. Differential DNA methylation was not observed among groups at the 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2) gene promoter. Significant hypomethylation was observed in pre-eclampsia but not nIUGR placentae for steroidogenic genes, including CYP11A1 (exon 1; EOPET; −9.66%; P < 0.0001), and CYP17 (exon 1A promoter; EOPET; −6.30%; P < 0.0001), 3β-hydroxy-delta-5-steroid dehydrogenase type 1 (HSD3B1 exon 2; EOPET; −12.49%; P < 0.0001), and LOPET; −6.88%; P < 0.001), TEA domain family member 3 (TEAD3 intron 1; EOPET; −12.56%; P < 0.0001) and CYP19 (placental-specific exon 1.1 promoter; EOPET; −10.62%; P < 0.0001). These data represent dysregulation of the placental epigenome in pre-eclampsia related to genes involved in maintaining the hormonal environment during pregnancy and highlights particular susceptibility in the early onset syndrome.

Introduction

Maternal stress and/or elevated glucocorticoid exposure in utero is associated with low birth weight, exaggerated infant stress response and adult disease in the offspring [1]. Disorders during pregnancy which alter maternal physiology and induce systemic inflammatory response are likely to augment stress-signalling pathways, and may be associated with adverse developmental programming. Pre-eclampsia, particularly that of early onset, is largely the result of poor placentaion, leading to hypoxic and inflammatory insult to surrounding tissue as well as under-perfusion of essential materials from mother to fetus [2–4]. Further maternal complications can incude necrosis of the liver, respiratory distress syndrome and seizures of eclampsia [5]. Clinically, pre-eclampsia is a common obstetrical complication in developed countries and remains a leading cause of maternal death [6], however, its actiology is poorly understood. In this study we aim to determine how placental stress-pathways might be altered in pre-eclampsia and related pregnancy complications. Cortisol is responsible for the physiological ‘stress response’, mediated through binding to the glucocorticoid receptor (GR) gene (nuclear receptor subfamily 3, group C, member 1 [NR3C1]) [7]. The release of cortisol may be triggered by a number of stimulatory cues to regulate immune response, cardiovascular function, metabolism and reproductive function [8]. Corticotropin releasing hormone (CRH) initiates this stress response through the hypothalamic pituitary adrenal (HPA) axis. Large quantities of placental CRH, identical to the hypothalamic form, are synthesised and secreted by the syncytiotrophoblast [9,10] and stimulate both the maternal and fetal pituitary to release adrenocorticotropin hormone (ACTH), thus inducing adrenal cortisol secretion [11–13]. In the placenta, CRH has multiple roles including the stimulation of estrogen synthesis [14] and increasing the availability of maternal glucose for placental uptake [15]. In contrast to hypothalamic CRH control, cortisol positively stimulates CRH

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* E-mail: wrobinson@cfri.ca

Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada,
production in the placenta [16,17]. CRH activity is controlled by sequestration to plasma CRH binding protein (CRHBP), thus regulating the levels of bioactive free CRH [13].

In the placenta, cortisol is inactivated by 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), encoded for by HSD11B2 [18]. Dysregulation of HSD11B2 in the placenta is linked to the detrimental actions of cortisol in offspring and is accompanied by low birth weight [19–22]. Pregnancy complications, including pre-eclampsia [23,24] and intrauterine growth restriction (IUGR) [25–27] have been associated with reduced placental activity/expression of HSD11B2.

Epigenetics is commonly studied in the context of developmental programming and DNA methylation is the most studied epigenetic mark [29]. The placenta largely contributes to the fetal environment through its endocrine and metabolic functions, in addition to nutrient exchange. While this alone can influence epigenetic susceptibility in the fetus, the placenta is also a target of environmental effects (reviewed in [29]). Illustrative of potential placental susceptibility to stress in utero, Wywolli et al. recently reported altered methyl donor components, including folate, methionine and choline in the placenta of rats exposed to glucocorticoids [30].

DNA methylation of the NR3C1 exon 1F promoter is widely studied, where altered methylation is observed in response to stress stimuli in rat [31] and human [32–37] studies across multiple tissues. DNA methylation is also proposed to regulate tissue-specific HSD11B2 expression [38,39]; and has been associated with placental HSD11B2 gene regulation in prenatally stress-exposed rats [40]. In addition, CRH mRNA expression in the human placenta is susceptible to increased maternal intake of the methyl donor choline, which is associated with increased DNA methylation of the CRH promoter [41].

The aim of this study was to assess the impact of placental pathology, including pre-eclampsia and normotensive (n)IUGR cases, on the DNA methylation status of several genes that are linked to stress-induced pathways. For this purpose, pre-eclampsia was further subdivided into two categories: those patients with early onset symptoms arising <34+0 weeks gestation (early onset pre-eclampsia [EOPET]), or arising ≥34+0 weeks gestation (late onset pre-eclampsia [LOPET]), the former of which carries a considerably worse maternal and fetal prognosis [5]. A candidate gene approach was undertaken through analysis of Illumina Infinium HumanMethylation450 BeadChip array data targeting >450,000 CpG sites across the human genome in a control and EOPET subset, followed by confirmation and extension to additional cohorts using bisulfite pyrosequencing.

Genes selected for analysis included those involved in 1) cortisol signalling: NR3C1, CRH, and CRH receptor (CRHR1) and pro-opiomelanocortin (POMC [ACTH precursor]), 2) cortisol bioavailability: CRHBP and HSD11B2, and 3) placental hormonal signalling: StAR-related lipid transfer domain protein 3 (STARD3), CYP11A1, 3β-hydroxy-delta-5-steroid dehydrogenase type 1 (HSD3B1), TEA domain family member 3 (TEAD3) and CYP19. Herein, we show the remarkable DNA methylation plasticity of many genes involved in stress and hormone pathways associated with the severe placental dysfunction present in EOPET.

Materials and Methods

Ethics Statement

This study was approved by the University of British Columbia and the Children’s & Women’s Health Centre of British Columbia. Women between the ages of 18 and 42 years were recruited from the BC Women & Children’s Hospital, Vancouver, during the second trimester of pregnancy following informed written consent.

Subjects

Cases included in this study partially overlap other studies published by our group [42–45]. Control cases consisted of normotensive women with no known placental pathology. In order to collect control samples at gestational ages matching that of case cohorts, premature deliveries involving a range of causes such as premature rupture of membranes, chorioamnionitis and cervical incompetence were included. Pre-eclampsia was defined using the Society of Obstetricians and Gynaecologists of Canada guidelines [46] which include the following criteria: 1) hypertension (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg measured twice, at least 4 hours apart) after 20 weeks gestation in combination with proteinuria (≥0.3 g/d or ≥2+ dipstick proteinuria) or another adverse condition (non-hypertensive and non-proteinuric haemolysis, elevated liver enzymes, low platelet count (HELLP) syndrome, fetal IUGR; or absent or reversed end-diastolic flow in the umbilical artery by Doppler velocimetry). EOPET was defined as symptoms arising <34+0 weeks and LOPET as symptoms arising ≥34+0 weeks gestation [5]. nIUGR was defined as either: birth weight below the 3rd percentile for gender and gestational age using Canadian population parameters [47], or birth weight below the 10th percentile in combination with a) persistent uterine artery notchng at 22+0 to 24+6 weeks, b) absent or reversed end diastolic velocity on umbilical artery Doppler, and/or c) oligohydramnios (amniotic fluid index <50 mm) and absent maternal hypertension. Gestational diabetes, isolated maternal hypertension, non-singleton pregnancies, still birth or fetal genetic anomaly cases were excluded from the study.

Patient characteristics for the full cohort are outlined in Table 1. As expected, birth weight was significantly lower in all case groups compared to controls. Furthermore, IUGR co-existed with pre-eclampsia in 14/19 EOPET and 5/18 LOPET cases. Gestational age was not matched in pre-eclampsia and control cases and was assessed as a cofounder in DNA methylation studies. Where possible, births were defined by the presence (spontaneous or induced vaginal delivery) or absence (elective caesarean section) of labour (Table 1). Emergency caesareans, or cases in which no data were available, were undefined. Labour was therefore defined in 54/111 controls, 10/19 EOPET, 12/18 LOPET and 9/13 nIUGR patients. Of these cases there was unequal distribution of labour/no labour births in EOPET and nIUGR patients compared with controls (χ²; P<0.001, P<0.05, respectively). Self-reported ethnicity was available in only a minority of cases, precluding evaluation of this as a separate variable. Of these, the majority reported Caucasian (~60%) or Asian (~30%), with the remainder of other or mixed ancestry.

Selection of Candidate Genes

A candidate gene approach was used to select a set of genes known to be directly involved in stress hormone signalling, availability and downstream actions, such as hormone production. Data were collected from an Illumina HumanMethylation450 BeadChip array (Illumina Inc., San Diego, CA); genome-wide results are being published separately by our group (Blair et al., in preparation; GEO: GSE44667). Briefly, tissue (1 cm³) was sampled from three chorionic villus sites and DNA extracted using standard salting out methods. Equal amounts of placental DNA were pooled from all three sites per placenta and bisulfite converted using the EZ DNA methylation kit (Zymo Research Corp., Irvine, CA) following the manufacturer’s instructions. A subset of
gestational age and gender matched control (n = 19) and EOPET (n = 19) placental DNA samples were run on the Infinium HumanMethylation450 BeadChip array according to the manufacturer’s protocol. Raw data were read using GenomeStudio (Illumina Inc.), colour-corrected [48], and batch-normalised. Correction for probe type using SWAN [49] and removal of probes targeting known SNPs was also performed [50]. Output data were reported as β values, representing DNA methylation at each locus within a range of 0–1. Each candidate gene was associated with a range of 3 to 22 CpG probes, except NR3C1 for which 37 associated CpG probes were located on the array. These represented both upstream (enhancers and promoters) and gene body regions. Significant differences in candidate gene DNA methylation between groups were identified by any associated variability (5% [51], candidate CpG sites were selected for further analysis, to allow for a more proximate location with respect to promoter or CpG island proximity was also considered. In the case of NR3C1 isoform-specific Polymerase Chain Reaction (PCR) assay 1 and 2, Gene expression data were obtained using a subset of gestational age and gender matched control (n = 8) and EOPET (n = 8) cases that were run on the Illumina DNA methylation array. RNA was extracted from one chorionic villus site stored at −80°C in RNAlater (Invitrogen), using the RNaseasy kit (Qiagen Ltd.) following the manufacturer’s protocol. RNA was assessed for quality using the Bioanalyzer 2100 (Agilent, Santa Clara, CA), reverse transcribed to cDNA (TotalPrep RNA Amplification kit, Illumina, Inc.) and hybridised to a HT-12v4 Expression BeadChip (Illumina, Inc.) according to the manufacturer’s protocol. This chip interrogates >47,000 transcripts genome-wide. Raw data were quantile normalised using GenomeStudio 2011 (Illumina, Inc.) and probes with bad detection P-values were removed and replaced with a value of zero. The full data set is being published separately [Blair et al., in preparation; GEO: GSE44711]. Expression data were available for all genes followed up in this study.

**Table 1.** Patient characteristics for cases assessed by bisulfite pyrosequencing.

| Parameter                  | Controls n = 111 | EOPET n = 19 | LOPET n = 18 | nIUGR n = 13 |
|----------------------------|------------------|--------------|--------------|--------------|
| Birth weight (g)           | 2590±989         | 1399±621     | 2742±798     | 2132±513     |
| Birth weight (SD)          | 0.06±0.07        | −1.51±0.22***| −0.73±0.20** | −1.67±0.20***|
| Co-existing IUGR           | –                | 14/19        | 5/18         | –            |
| Gestational age (weeks)    | 35.09±4.21       | 31.93±3.27** | 37.47±2.29*  | 36.43±2.31   |
| Maternal age (years)       | 33.10±4.74       | 34.16±6.01   | 33.49±5.47   | 34.64±5.26   |
| Female: Male ratio         | 55:56            | 7:12         | 10:8         | 9:4          |
| Mode of delivery           |                  |              |              |              |
| Labour                     | 46               | 4            | 9            | 5            |
| No Labour                  | 8                | 6            | 3            | 4            |
| Undefined                  | 57               | 9            | 6            | 4            |

Birth weight statistics are based on standard deviation (SD) points relative to gestational age and gender matched normal birth weight ranges. Gestational age values are given at birth. Data are mean ± SD. The mode of delivery is characterised as ‘Labour’ (spontaneous or induced vaginal delivery), ‘No Labour’ (elective caesarean section) or, ‘Undefined’: emergency caesarean section or unknown. EOPET: early onset pre-eclampsia, LOPET: late onset pre-eclampsia, nIUGR: normotensive intrauterine growth restriction, 

*P<0.05, **P<0.01, ***P<0.001, compared to controls.

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**Bisulfite Pyrosequencing**

To assess CpG methylation in our extended cohort of controls and cases, placental DNA samples were analysed using bisulfite pyrosequencing. Forward, reverse and sequencing primers for bisulfite converted DNA were designed with the aid of Pyro Q-CpG software (Qiagen Ltd., GmbH, Hilden, Germany; Table S1). PCR reactions consisted of ~20 ng bisulfite-converted DNA, 1× PCR buffer (Qiagen Ltd.), 0.2 mM dNTPs (Invitrogen, Carlsbad, CA), 0.4 μM forward and reverse primers (Integrated DNA Technologies Inc., Coralville, IA) and 0.18 U DNA polymerase (HotStarTaq, Qiagen Ltd.). Optimised PCR conditions consisted of 95°C for 15 min, 40 cycles of 95°C for 30 sec, variable annealing temperature (Ta) for 30 sec, 72°C for 30 sec, and finally 72°C for 10 min. The optimal Ta was 51°C for CYP19, 55°C for NR3C1 assay 1 and 2, CRHBP, CYP11A1, HSD3B1, HSD11B2 and TEAD3, and 57°C for CRH PCR reactions. PCR products were sequenced and DNA methylation analysed at each CpG site using the Pyromark Q96 MD Pyrosequencer (Qiagen Ltd.). Samples from each patient group were equally allocated to multiple pyrosequencing plates to cancel out biases due to inter-plate variability (<5% for each assay). Where possible, more than one CpG site was included in the pyrosequencing assay, in addition to the site identified using the DNA methylation array.

**Gene Expression Array**

Gene expression data were obtained using a subset of gestational age and gender matched control (n = 8) and EOPET (n = 8) cases that were run on the Illumina DNA methylation array. RNA was extracted from one chorionic villus site stored at −80°C in RNAlater (Invitrogen), using the RNaseasy kit (Qiagen Ltd.) following the manufacturer’s protocol. RNA was assessed for quality using the Bioanalyzer 2100 (Agilent, Santa Clara, CA), reverse transcribed to cDNA (TotalPrep RNA Amplification kit, Illumina, Inc.) and hybridised to a HT-12v4 Expression BeadChip (Illumina, Inc.) according to the manufacturer’s protocol. This chip interrogates >47,000 transcripts genome-wide. Raw data were quantile normalised using GenomeStudio 2011 (Illumina, Inc.) and probes with bad detection P-values were removed and replaced with a value of zero. The full data set is being published separately [Blair et al., in preparation; GEO: GSE44711]. Expression data were available for all genes followed up in this study.

**NR3C1 Isoform-specific Polymerase Chain Reaction (PCR)**

NR3C1 exon 1C and exon 1D isoform-specific PCR was performed across five chorionic villus samples obtained over different gestational time points, including 7, 19, 24, 32 and 40 weeks of gestation. RNA was extracted from tissue samples stored at −80°C in RNAlater using the phenol-chloroform method.
Commercially available adult male hippocampus RNA was used as a positive control for the \(NR3C1\) exon 1D expression (BioChain Institute, Inc., Newark, CA). RNA concentrations and purities were measured using the Nanodrop 1000 spectrophotometer (ThermoScientific, Wilmington, DE). cDNA was prepared from 200 ng RNA using the High Capacity RT kit (Applied Biosystems). PCR primer sequences were exon-exon spanning \(NR3C1\) exon 1D forward primer 5'-ACCGCTAAACCACAGAACAC-3', \(NR3C1\) exon 1C forward primer 5'-GGGGCATGGGAGAGTCTTTCTT-3', and a shared \(NR3C1\) exon 1C/D reverse primer 5'-TCCATCACTATCCTCCCCCTCTC-3'. Amplicon length for \(NR3C1\) exon 1C and 1D transcripts were 288 and 159 base pairs, respectively. PCR reactions were prepared as described in section 2A, using 1 uL cDNA and a Ta of 60°C for both assays. PCR products were run on a 1% agarose gel at 120 V for 45 min, alongside a 100 base pair DNA ladder (Invitrogen) and visualised using a Universal Hood II gel imager and Quantity One v4.6.9 software (Bio-Rad, Hercules, CA).

**Statistics**

Patient clinical data were compared using a Student’s t-test, non-parametric Mann-Whitney test or a chi-squared test where appropriate. DNA methylation array data for control and EOPET cases were compared using a Mann-Whitney test. For bisulfite pyrosequencing analyses, Spearman’s Rho correlation coefficients were calculated to determine CpG correlation within an assay. Univariate linear regression analyses were performed in control and case cohorts to identify potential confounding factors that influence mean DNA methylation at a selected gene. Variables assessed included birth weight, gestational age at birth, maternal age, gender of infant and the presence/absence of labour. If confounding factors were absent, an analysis of covariance correcting for that variable was applied. All analyses included a Levene’s test for equality of error variances and were followed by Bonferroni post-hoc tests for multiple comparisons. RNA expression array data for control and EOPET cases were compared using a Student’s t-test or Mann-Whitney test as appropriate. \(P\) values of <0.05 were considered significant.

**Results**

**Candidate Genes for Differential Placental DNA Methylation in Pre-eclampsia**

Multiple genes associated with stress pathways and steroid production were associated with differentially methylated CpG sites in EOPET cases compared with controls based on the Illumina DNA methylation array (Table 2). In the region containing alternative \(NR3C1\) exon 1s and respective upstream promoters, differential DNA methylation was observed at CpG sites adjacent and upstream of exon 1D (Fig. 1). In EOPET placentae there was significant hypermethylation of these sites compared to controls (Fig. 1; Table 2). Interestingly, no other \(NR3C1\) promoter regions, including the widely studied exon 1F region, were altered in EOPET placentae. In fact, in normal placenta CpG sites across the \(NR3C1\) promoter region were largely unmethylated (\(P\) value 0–0.1), with the exception of exon 1D in which DNA methylation values were greater (\(P\) value 0.1–0.3; Fig. 1).

Regions associated with other candidate genes also exhibited altered placental DNA methylation patterns in control versus EOPET cases (Table 2). A CpG island associated with \(CRHBP\) (Fig. S1) was significantly hypermethylated in EOPET, whereas non-CpG island regions associated with \(NR3C1\), \(CRH\), \(HSD3B1\), \(TEAD3\) and \(CYP19\) were hypomethylated in EOPET versus control placentae (Fig. S2-5, respectively). A CpG associated with the transcription factor \(TEAD3\) was particularly altered, with an average \(\beta\) value decrease of 0.21 in EOPET (\(P<0.0001\)). DNA methylation values between control and EOPET groups were altered by \(>0.1\), or 10% points, at CpG sites within steroidogenic pathway genes including \(CYP11A1\), \(HSD3B1\) and \(CYP19\) (all \(P<0.0001\)). Candidate genes that were assessed but revealed no alteration in placental DNA methylation patterns at individual CpG sites in control and EOPET cases included \(CRHR1\), \(HSD11B2\), \(POMC\), \(STARD3\) and \(CYP17\) (data not shown).

Bisulfite pyrosequencing was performed to confirm DNA methylation differences at significant CpG sites in a larger cohort of controls as well as cases of LOPET and nIUGR. For control and EOPET samples that were assessed by both array and pyrosequencing techniques, DNA methylation values at investigated CpG sites for the two methods were highly correlated (all loci Spearman’s Rho 0.8; \(P<0.001\)). Within pyrosequencing assays, DNA methylation values at individual CpG sites were also correlated (Fig. S7), and therefore mean methylation values for each region were compared.

**Gestational Age, but not Presence/absence of Labour, is a Confounder of Placental DNA Methylation at Candidate Genes**

Using the bisulfite pyrosequencing data, univariate linear regression analyses for each studied region were performed on control and case cohorts to determine whether specific known patient variables might influence DNA methylation in that cohort. Birth weight, maternal age and infant gender were not significant confounders in any group assessed (data not shown). Gestational age significantly influenced DNA methylation in third trimester control placentae (\(n=111\); range 28–40 weeks) in a gene dependent manner. Increasing gestational age was negatively associated with CpG methylation within targeted regions of \(CRH\) (\(R = 0.19; P<0.05\), \(CYP19\) (\(R = 0.27; P<0.01\), \(TEAD3\) (\(R = 0.51; P<0.0001\)) and \(HSD3B1\) (\(R = 0.36; P<0.001\)) genes, and positively associated with CpG methylation of \(CRHBP\) (\(R = 0.26; P<0.01\)). However, there was no significant effect of gestational age on CpG methylation of the \(NR3C1\) exon 1D promoter region (\(R = 0.03\) nor \(CYP11A1\) (\(R = 0.13\)). Consequently, gestational age was corrected for in those genes in which it was found to be a confounder. It should be noted that even though the EOPET cases were on average of an earlier gestational age than controls, the methylation trends in EOPET were generally in the opposite direction than expected based on these gestational age associations.

Since the process of labour can induce gene expression changes in the placenta [53,54], we assessed whether or not labour affected DNA methylation at the genes included in this study. In a clearly defined subset of control patients, those that experienced labour (\(n=46\)) and those that did not (\(n=8\)), were compared. Although sample size in the non-labour group was small and mode of delivery data were not available for the full cohort, preliminary data suggested no significant DNA methylation changes at any of the genes/CpG sites studied as a result of labour (data not shown). As changes in DNA methylation may be dependent on cell replication, labour would not be expected to be associated with large changes in DNA methylation, and this confirms that the...
Figure 1. CpG sites on the Illumina Infinium HumanMethylation450 BeadChip array across the NR3C1 gene region. Upper graph: DNA methylation at each CpG site in control (n = 19) and early onset pre-eclampsia (EOPET; n = 19) placentae. Lower graph: enlargement of region associated with CpG island containing multiple alternative first exons (black boxes). The Illumina CpG probe identifier is indicated by cg# and the position of CpG sites on the graphs are not to scale. *P<0.05.
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Table 2. Illumina Infinium HumanMethylation450 BeadChip array data.

| Gene     | Chromosome location | CpG of interest | Gene region          | CpG island? | DNA methylation (β value) | Δ β | P-value |
|----------|---------------------|-----------------|-----------------------|-------------|--------------------------|-----|---------|
| NR3C1    | ch5 (q31.3)         | cg14558428      | exon 1D promoter      | Y           | 0.14±0.08                | 0.23±0.12 | 0.09 | 0.01 |
| NR3C1    | ch5 (q31.3)         | cg24026230      | exon 1D promoter      | Y           | 0.11±0.06                | 0.19±0.13 | 0.08 | 0.02 |
| CRH      | ch8 (q13.1)         | cg23049074      | exon 1 (5’ UTR)       | N           | 0.37±0.12                | 0.29±0.06 | −0.08 | 0.03 |
| CRHBP    | ch5 (q13.3)         | cg13777717      | intron 3              | Y           | 0.40±0.08                | 0.48±0.07 | 0.08 | 0.001 |
| CYP1A1   | ch15 (q24.1)        | cg06285340      | exon 1                | N           | 0.54±0.03                | 0.42±0.04 | −0.12 | <0.0001 |
| HSD3B1   | ch1 (p12)           | cg16175792      | exon 2                | N           | 0.82±0.02                | 0.69±0.05 | −0.13 | <0.0001 |
| TEAD3    | ch6 (p21.31)        | cg10893014      | intron 1              | N           | 0.60±0.06                | 0.39±0.10 | −0.21 | <0.0001 |
| CYP19    | ch15 (p21.2)        | cg15329467      | placental-specific exon 1.1 promoter | N | 0.56±0.05 | 0.46±0.05 | −0.10 | <0.0001 |

The Illumina CpG of interest, gene location and CpG island (defined as length >200 base pairs, 50% GC content and >0.6 CG ratio) association is provided. Mean ± SD methylation (β values and P-values are given for each gene for control (n = 19) and early onset pre-eclampsia (EOPET; n = 19) placental samples. NR3C1: nuclear receptor subfamily 3, group C, member 1, CRHBP: corticotropin releasing hormone (CRH) binding protein; HSD3B1: 3β-hydroxy-delta-5-steroid dehydrogenase type 1, TEAD3: TEA domain family member 3.
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methylating changes we observed are unlikely to be altered by short term exposures at the end of pregnancy.

Glucocorticoid Signalling Pathway Genes are Differentially Methylated in Pre-eclampsia

Differential placental DNA methylation of *NR3C1*, proximal to the exon 1D promoter, was verified in EOPET cases at multiple CpG sites by bisulfite pyrosequencing. Average DNA methylation values were significantly increased across the *NR3C1* exon 1D region compared with controls (+8.4%; P<0.01; Fig. 2A). In addition, there were trends indicative of increased DNA methylation at this locus in LOPET placenta relative to controls (+6.4%; P=0.09; Fig. 2A). Increased promoter methylation of *NR3C1* could be a feature of both early and late pre-eclampsia, but perturbations are more pronounced in the earlier onset cases. Alternatively, a subset of cases classified as LOPET may have a placental aetiology more similar to EOPET, causing a trend towards similar changes. Increased promoter methylation of *NR3C1* could be a feature of both early and late pre-eclampsia, but perturbations are more pronounced in the earlier onset cases. Alternatively, a subset of cases classified as LOPET may have a placental aetiology more similar to EOPET, causing a trend towards similar changes. While *NR3C1* DNA methylation trends for nIUGR cases appeared similar to that of LOPET placenta, mean methylation differed from controls by <5% and was not significantly altered (Fig. 2A).

Genes involved in the hormonal regulation of glucocorticoid levels were also followed up with bisulfite pyrosequencing. In line with DNA methylation array findings, there was a non-significant trend for decreased CpG methylation at the *CRH* gene in EOPET placenta (5′ UTR; −4.30%; P=0.11; Fig. 2B) and an opposing significant increase in DNA methylation associated with *CRHBP* (intron 3; +9.14%; P<0.05; Fig. 2C), compared with controls. However, DNA methylation at these CpG sites was not significantly altered in LOPET or nIUGR placentae relative to controls.

Steroidogenic Pathway Genes are Differentially Methylated in Pre-eclampsia

The shift in DNA methylation at Illumina CpG sites associated with steroidogenic genes was confirmed in pre-eclampsia-associated placenta by bisulfite pyrosequencing. In EOPET there were significant losses of DNA methylation at regions associated with *CYP11A1* (exon 1; −9.66%; P<0.00001; Fig. 3A) and *HSD3B1* (exon 2; −12.49%; P<0.00001; Fig. 3B) compared with controls. In addition, the same loci were perturbed in LOPET cases (*CYP11A1*: −5.77%; P<0.001 and *HSD3B1*: −6.88%; P<0.001). *TEAD3* was significantly hypomethylated in EOPET compared with controls (intron 1; −12.56%; P<0.00001), but not in LOPET placenta (Fig. 3C). Similarly, hypomethylation of CpG sites associated with *CYP19* in EOPET cases (exon 1.1; −10.6%; P<0.00001; Fig. 3D) was not present in LOPET cases compared with control placentae. In addition, there was no significant association of isolated nIUGR with placental DNA methylation found for any of the steroidogenic genes studied (Fig. 3).

DNA Methylation of the HSD11B2 Promoter is not Altered in nIUGR

We elected to assess *HSD11B2* promoter DNA methylation by bisulfite pyrosequencing in our nIUGR cohort, targeting 11 consecutive CpG sites including four CpG sites previously

![Figure 2. Placental DNA methylation of genes involved in cortisol signalling and bioavailability.](https://example.com/image.png)

DNA methylation at CpG sites within A) exon 1D promoter of the nuclear receptor subfamily 3, group C, member 1 (*NR3C1*), B) corticotropin releasing hormone (*CRH*) and C) CRH binding protein (*CRHBP*) genes. Control, early onset pre-eclampsia (EOPET), late onset pre-eclampsia (LOPET) and normotensive intrauterine growth restriction (nIUGR) placenta are compared. Median and interquartile ranges are given based on average assay CpG methylation measured by bisulfite pyrosequencing. *P<0.05, **P<0.01.

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associated with birth weight across normal ranges and increased DNA methylation in IUGR cases [52]. Methylation values at different CpG sites across this region were variable and were not correlated (Fig. S7); therefore DNA methylation was assessed at individual CpG sites. Univariate linear regression analyses revealed no confounding effect of gestational age, maternal age, infant gender or mode of delivery (data not shown). Also, there was no significant relationship between birth weight and HSD11B2 promoter DNA methylation across normal birth weight ranges in our cohort of 111 controls (Table S2). Furthermore, we did not observe altered HSD11B2 promoter methylation between control and nIUGR cases (Fig. 4A and B). Pyrosequencing for EOPET and LOPET cohorts also confirmed no change in DNA methylation values at this locus in pre-eclampsia (Fig. 4A and B), as previously indicated by DNA methylation array data.

DNA Methylation is Inversely Associated with mRNA Expression for Selected Genes

Gene expression array data were available for a small subset of matched control and EOPET cases overlapping the larger cohort. Significant upregulation of CRH (≥20 fold), CYP11A1 (4 fold), HSD3B1 (5 fold) and CYP19 (5 fold) transcripts was observed in EOPET cases compared to controls (Table 3; all P<0.01) but not TEAD3. Also, there were trends for the downregulation of CRHBP and upregulation of HSD11B2 expression in EOPET-associated placentae (Table 3). The expression of NR3C1 mRNA was not altered; however, as the probe to detect NR3C1 mRNA was not transcript-specific, it is possible that subtle changes in isolated transcript expression would not be detected.

In addition, gene expression across both control and EOPET groups was inversely correlated with DNA methylation in promoter regions of CRHBP (R = −0.70, P<0.01), CYP11A1 (R = −0.76; P<0.01), HSD3B1 (R = −0.55; P= 0.05) and CYP19 (R = −0.36) genes, although not CRH (R = −0.05).

The NR3C1 Exon 1D Transcript is Expressed in Placenta Across Gestation

Isoform-specific PCR was performed for NR3C1 exon 1C and exon 1D to determine their expression in the placenta over gestation (Fig. 5). The ubiquitously expressed exon 1C was observed across all gestational ages assessed including 7, 19, 24, 32 and 40 week gestationally aged placentae samples. The exon 1D transcript was also expressed in the placenta across these developmental stages. Since this transcript has been previously reported only in the adult hippocampus, and absent from a range of other tissues [55], expression of NR3C1 exon 1D may be of particular importance in the placenta.

Discussion

The majority of normotensive pregnancies are characterised by the absence of placental cortisol; however, cortisol is detectable in the placenta of up to 80% of pre-eclampsia-associated pregnancies [24]. This is of concern given the overwhelming evidence for the role of glucocorticoids in the developmental programming of disease. This is documented in both animal and human studies,
and is associated with low birth weight, hypersensitive stress response and neurobehavioural anomalies in infants, and increased risk of metabolic, cardiovascular and reproductive syndromes in adults (reviewed in [1,56,57]). Determining the types of pregnancies that are at elevated risk for such programming events and understanding the mechanisms that lead to these changes may permit future advancements in obstetrical and neonatal care and improved long-term health outcomes.

The human NR3C1 gene contains multiple tissue-specific alternative first exons, each containing upstream promoters that can be silenced by DNA methylation [55,58]. Previously, altered DNA methylation at specific CpG sites within the exon 17 promoter in rat hippocampi [31,59], and in the human orthologue exon 1F in brain, peripheral and cord blood, and placenta [32,34,35,37], has been associated with perinatal or prenatal stress. In the placenta, DNA methylation at NR3C1 exon 1F promoter is associated with neurological and mental health outcomes in infants in normal pregnancy [36] and in babies large for gestational age [60]. The biological impact of the small DNA methylation changes (1–5% points) reported at the exon 1F promoter, a region exhibiting very low levels of methylation (<10%), is not clear.

In this study, promoter-wide DNA methylation profiling revealed differential patterns at the exon 1D promoter, but not at exon 1F. Levels of DNA methylation in the exon 1D region ranged from 5–40% in normal placenta, agreeing with previous

Figure 4. Placental DNA methylation of the 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2) promoter. Control, early onset pre-eclampsia (EOPET), late onset pre-eclampsia (LOPET) and normotensive intrauterine growth restriction (nIUGR) placentae are compared. Mean ± SD DNA methylation values are given for consecutive CpG sites in HSD11B2 assay 1 (A) and assay 2 (B) measured by bisulfite pyrosequencing. doi:10.1371/journal.pone.0062969.g004

Table 3. Illumina HT-12v4 Expression BeadChip gene expression array data for genes followed up by bisulfite pyrosequencing.

| Gene    | Array probe | RNA expression (Fluorescent Units) | P-value |
|---------|-------------|-----------------------------------|---------|
|         |             | Control | EOPET |         |
| NR3C1   | ILMN_2389347| 59.4±16.3 | 57.8±12.2 | NS |
| CRH     | ILMN_1668035| 64.2±20.6 | 1431±1124 | <0.01 |
| CRHBP   | ILMN_1761312| 153.9±137.6 | 90.7±90.5 | NS |
| CYP11A1 | ILMN_1768820| 442.8±277.2 | 1750±1377 | <0.01 |
| HSD3B1  | ILMN_1780693| 238.4±120.2 | 1193±857 | <0.01 |
| TEAD3   | ILMN_1814002| 118.9±44.9 | 119.9±38.8 | NS |
| CYP19   | ILMN_2387860| 112.0±54.6 | 671.4±579.8 | <0.01 |
| HSD11B2 | ILMN_1813350| 37.4±21.5 | 148.6±294.2 | NS |

The Illumina probe identifier, mean ± SD expression values and the associated P-value is given for each gene for a subset of control (n = 8) and early onset pre-eclampsia (EOPET; n = 8) placental samples. NR3C1: nuclear receptor subfamily 3, group C, member 1, CRHBP: corticotropin releasing hormone (CRH) binding protein; HSD3B1:3β-Hydroxy-delta-5-steroid dehydrogenase type 1, TEAD3: TEA domain family member 3, HSD11B2:11β-hydroxysteroid dehydrogenase type 2. doi:10.1371/journal.pone.0062969.t003
reports of large interindividual variation within the NR3C1 promoter [61]. While this interindividual variation was also present in pathological placental, DNA methylation values were on average significantly higher than normal placentae, providing evidence that at least in the human placenta, exon 1D may be more susceptible to environmental insults than alternative promoter regions. The interrogated CpG sites were proximal to the putative core sequence (CGCCATnTT) for the ubiquitously expressed zinc finger transcription factor Yin Yang 1 (YY1), in addition to various other transcription factor motifs located in the exon 1D promoter [61]. Given the proximity of these consensus sequences to regions of differential DNA methylation in our study, we speculate that altered DNA methylation may be representative of an altered transcriptional landscape in these cases. In addition to gene expression being transiently influenced by events in the perinatal period, such as mode of delivery, it can also be affected by placental processing time; while such factors have not been shown to have major effects on DNA methylation [62].

Given the complexity of NR3C1 gene regulation and the fact that alternative splice variants are expressed in the placenta before and after labour [54], we wished to determine if the differentially methylated promoter, exon 1D, was expressed in the placenta. Previous reports indicated that exon 1D was exclusively expressed in adult hippocampal tissue and was absent from a panel of adult peripheral tissues and cells [55]. We found that NR3C1 exon 1D is expressed in the human placenta over gestational ages ranging from the first to the third trimester. Of interest, this promoter region exclusively maps to histone-3 lysine-4 mono-methylation ‘active’ marks across numerous cell lines, is predicted to be within a strong enhancer region according to ENCODE and is contained within a DNase I hypersensitivity cluster. These observations suggest a regulatory role for the promoter region upstream of exon 1D that could be involved in overall NR3C1 expression.

During development the HPA axis is susceptible to altered programming, of which the CRH signalling system is a target [21]. In rats, in utero exposure to stress elevates cortisol in offspring, which is associated with increased hypothalamic CRH expression [63], and decreased CRHBP and increased ACTH plasma concentrations [64, 65], indicating increased adrenal cortisol production and bioavailability. Like the hypothalamic isoform, placental CRH can stimulate maternal and fetal adrenal steroidogenesis, as well as placental hormone production, specifically repressing progesterone [66] and augmenting estrogen [14] synthesis in trophoblasts.

In women with high third trimester choline supplementation, a 4–5% increase in DNA methylation at CpG sites within the CRH promoter in placenta was associated with a ~33% fall in placental CRH transcript levels and cord blood cortisol concentration, suggesting a direct effect on the fetal HPA axis [41]. As these DNA methylation changes were observed at CpG sites adjacent to the sites included in our study, an equivalent decrease in DNA methylation levels in EOPET compared to controls may reflect increased transcript levels. Indeed, placental CRH content and umbilical cord and maternal plasma CRH are significantly elevated in pre-eclampsia-associated pregnancies compared to normal pregnancies [67–71]. In this study, we further confirmed an increase in placental CRH mRNA in a subset of pre-eclampsia cases, although we did not observe a relationship between DNA methylation at these particular CpG sites and transcript expression in this small number of cases.

Elevated CRH has been reported in umbilical cord plasma of IUGR infants [72]. However, we found no change in CRH DNA methylation levels in placentae of nIUGR pregnancies in our study. While multiple regulatory processes in addition to DNA methylation can influence CRH gene expression, it is also possible that UGR cases utilised in other studies included cases with co-existing hypertension/pre-eclampsia that might have contributed to altered CRH levels. Increased CRHR1 gene expression was also reported in placentae of complicated pregnancies including pre-eclampsia and IUGR [73]; however, we did not detect altered DNA methylation of the CRHR1 gene in this study.

Intragenic DNA methylation can be associated with altered gene expression, and may regulate alternative promoters [74]. In EOPET-associated placentae, we observed hypermethylation of CRHBP at CpG sites contained within a CpG island overlapping the third intron. There was an inverse relationship between DNA methylation at this region and RNA expression across a subset of control and EOPET cases and a trend for downregulation of CRHBP mRNA in pre-eclampsia-associated placentae. Other studies have found reciprocal decreases in CRHBP and increases in CRH transcripts in the placenta [75] or protein levels in the plasma [70] of women who developed pre-eclampsia. Thus, elevated levels of circulating CRH, through increased placental CRH and/or decreased CRHBP production may be a mechanism to stimulate increased downstream glucocorticoid synthesis.

The physiological elevation of maternal glucocorticoids during the third trimester is normally counteracted by the concurrent upregulation of placental HSD11B2 [25]. Adverse effects of glucocorticoid exposure through the inhibition of placental HSD11B2 expression/11bHSD type 2 activity in offspring are well documented [19, 20, 22, 76], and have been associated with hypermethylation of CpG sites within the Hsd11b2 promoter in rats [40]. Despite lowered placental HSD11B2 expression being reported in pre-eclampsia [23, 24], DNA methylation patterns across the HSD11B2 promoter and gene regions were not altered in our cohort. Additionally, we found only a moderate trend for HSD11B2 mRNA downregulation in our sample subset. Further-

Figure 5. NR3C1 isoform expression in the human placenta. Expression of NR3C1 exon 1C and 1D alternative transcripts in the placenta across gestation. A cDNA reaction prepared from adult male hippocampus RNA was used as a positive control. The negative control consisted of a cDNA reaction omitting reverse transcriptase. NR3C1 exon 1C and 1D PCR amplions were 288 base pairs (bp) and 159 bp in length, respectively.

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more, there was no relationship between HSD11B2 promoter DNA methylation and birth weight, as indicated in a previous study [52].

To sustain considerable estrogen and progesterone synthesis required for placental function and pregnancy maintenance, the placental syncytiotrophoblast abundantly express steroidogenic pathway genes [77]. In the placenta, a structural homologue to steroidal acute regulatory protein, STARD3, transports cholesterol for conversion to pregnenolone by cytochrome P450 side chain cleavage (encoded by CYP11A1) [78,79]. DNA methylation of CpG sites at the promoters of STARD3 and CYP11A1 genes was assessed given their rate limiting involvement in placental steroidogenesis. Placental DNA methylation patterns associated with STARD3 were unaltered among cases; however, there was significant hypomethylation of CYP11A1 in pre-eclampsia cases compared with controls. Furthermore, our findings of upregulation of CYP11A1 in pre-eclampsia-associated placentae, and a negative correlation between placental CYP11A1 DNA methylation and RNA expression support a role for DNA methylation in regulating CYP11A1 expression. The transcription factor activating protein 2 (AP2) is proposed to transactivate CYP11A1 in the placenta [80] and the binding of this factor to gene promoters is sensitive to DNA methylation in other tissues [81]. Based on the DNA methylation array data, the differential DNA methylation observed in pre-eclampsia-associated placentae is likely to extend into this consensus sequence located 100 base pairs upstream of interrogated CpG sites.

The placental production of up to 250–300 mg progesterone per day is maintained by the conversion of pregnenolone into progesterone by 3β-hydroxy-δ5-steroid dehydrogenase type 1 (HSD3B1) [77]. Placental-specific enhancers are located 2500–3000 base pairs upstream of the HSD3B1 promoter, and contain a binding site for transcription enhancer factor 5 (TEFD3; known), which is predominantly expressed by the placenta [82] and necessary to confer placental-specific HSD3B1 expression [83]. Regions associated with both
association with *CYP19* (exon 2) and *HSD3B1* up-regulation of regulation of placental methylation may also play a prominent role in tissue-specific exon 1.1 promoter and transcript expression. Therefore, DNA for a negative association between was not altered, significant hypomethylation was observed (aromatase) were also studied. While DNA methylation of *17* methylated CpG region in EOPET was contained within the alternative first exons [84]. In this study, the differentially methylated CpG region in EOPET was contained within the placental-specific *CYP19* exon 1.1 promoter [85]. At other tissue-specific human *CYP19* promoters, DNA methylation has been associated with *CYP19* expression [86,87]. We found significant up-regulation of *CYP19* mRNA in EOPET placentae and a trend for a negative association between *CYP19* DNA methylation of the exon 1.1 promoter and transcript expression. Therefore, DNA methylation may also play a prominent role in tissue-specific regulation of placental *CYP19*; however a larger sample size is required to confirm this.

It was somewhat surprising that the altered DNA methylation found in cortisol-signalling and hormone production genes was limited to pre-eclampsia and not observed in nIUGR cases. Birth weight was not a predictor of DNA methylation in any of the genes/CpG sites included in this study. Pre-eclampsia was accompanied by IUGR in 14/19 EOPET and 5/18 LOPET cases; however, we did not find significant effects of IUGR within pre-eclampsia groups. It is important to include isolated IUGR cases as a control group to delineate the effects of fetal and placental growth restriction with that of pre-eclampsia. Furthermore, the majority of DNA methylation changes were limited to early onset rather than late onset disease, supporting the idea that EOPET is aetiologically distinct to LOPET and that the cut-off of 34 weeks can distinguish cases with relatively greater placental involvement, rather than manifestations of disease primarily in the mother [88-92]. However, there was some overlap, in which hypomethylation of *CYP11A1* and *HSD3B1* genes was present in LOPET cases. Certain changes may occur in response to common features such as exposure to anti-hypertensive medication, even if the underlying pathology is different. Changes unique to EOPET may reflect more fundamental aetiological differences.

We report altered DNA methylation proximal to genomic regions previously characterised as important for gene regulation, which is correlated with mRNA expression in some cases. These data may represent common dysregulation of stress and hormonal pathways in pre-eclampsia. The biological consequences of altered expression of cortisol signalling and endocrine genes in the placenta in pre-eclampsia are likely to be complex and are hypothetically modelled in Figure 6. Reduced placental GR activation in combination with elevated CRH output may stimulate both maternal and fetal adrenal cortisol synthesis [10,12] thus aberrantly increasing fetal exposure to glucocorticoids during development. Elevated CRH may also exert direct effects on placental function, including effects on glucose uptake and vascular tone and may contribute to the premature timing of labour common to pre-eclampsia [15]. While CRH stimulates placental estrogen synthesis (*CYP19* expression) [14], its repressive effect on progesterone synthesis (*CYP11A1* and *HSD3B1* expression) [66] may be dampened in the pre-eclampsia-associated placenta. Maternal progesterone, but not estrogen, concentrations are increased in women with preeclampsia [93,94] and both steroid hormones positively stimulate *CYP11A1* and *HSD3B1* expression in trophoblast cells [95]. Therefore elevated progester-one, despite elevated CRH, may act in a compensatory feed-forward loop to promote placental steroidogenesis.

In summary, we suggest that changes in DNA methylation may contribute to the altered expression of cortisol-signalling genes previously linked to pre-eclampsia, and exclude this mechanism as an important regulatory factor in other related pregnancy complications. In addition, we highlight previously uncharacterised genes involved in placental steroidogenesis as differentially methylated in pre-eclampsia. Additional functional characterisation of these genes is required to further assess the relationship between DNA methylation and gene expression in the human placenta and will form the basis of future experiments. Unravelling which genetic pathways are susceptible to epigenetic modification in the placenta may provide a clearer aetiological perspective in disease cases and basis for targeted intervention.

Supporting Information

Figure S1 CpG sites on the Illumina Infinium Human-Methylation450 BeadChip array across the *CRHBP* gene region. Upper panel: UCSC genome browser shot detailing location of Illumina CpG probes, histone marks predictive of enhancers, CpG island(s) and conserved transcription factor binding sites (TFBS) associated with the gene body. Lower panel: DNA methylation pattern at Illumina CpG sites across the corticotropin releasing hormone binding protein (*CRHBP*) gene region in control (n = 19) and early onset pre-eclampsia (EOPET; n = 19) placentae. The Illumina CpG probe identifier is indicated by cg# and the position of CpG sites on the graphs are not to scale. Values are mean ± SD, and *P*<0.05 based on Mann-Whitney tests. (DOCX)

Figure S2 CpG sites on the Illumina Infinium Human-Methylation450 BeadChip array across the *CRH* gene region. Upper panel: UCSC genome browser shot detailing location of Illumina CpG probes, histone marks predictive of enhancers, CpG island(s) and conserved transcription factor binding sites (TFBS) associated with the gene body. Lower panel: DNA methylation pattern at Illumina CpG sites across the corticotropin releasing hormone (*CRH*) gene region in control (n = 19) and early onset pre-eclampsia (EOPET; n = 19) placentae. The Illumina CpG probe identifier is indicated by cg# and the position of CpG sites on the graphs are not to scale. Values are mean ± SD, and *P*<0.05 based on Mann-Whitney tests. (DOCX)

Figure S3 CpG sites on the Illumina Infinium Human-Methylation450 BeadChip array across the *CYP11A1* gene region. Upper panel: UCSC genome browser shot detailing location of Illumina CpG probes, histone marks predictive of enhancers, CpG island(s) and conserved transcription factor binding sites (TFBS) associated with the gene body. Lower panel: DNA methylation pattern at Illumina CpG sites across the *CYP11A1* gene region in control (n = 19) and early onset pre-
eclampsia (EOPET; n = 19) placentae. The Illumina CpG probe identifier is indicated by cg# and the position of CpG sites on the graphs are not to scale. Values are mean ± SD, and *P<0.05 based on Mann-Whitney tests.

(DOCX)

Figure S4 CpG sites on the Illumina Infinium Human-Methylation450 BeadChip array across the HSD3B1 gene region. Upper panel: UCSC genome browser shot detailing location of Illumina CpG probes, histone marks predictive of enhancers, CpG island(s) and conserved transcription factor binding sites (TFBS) associated with the gene body. Lower panel: DNA methylation pattern at Illumina CpG sites across the 3β-hydroxy-delta-5-steroid dehydrogenase type 1 (HSD3B1) gene region in control (n = 19) and early onset pre-eclampsia (EOPET; n = 19) placentae. The Illumina CpG probe identifier is indicated by cg# and the position of CpG sites on the graphs are not to scale. Values are mean ± SD, and *P<0.05 based on Mann-Whitney tests.

(DOCX)

Figure S5 CpG sites on the Illumina Infinium Human-Methylation450 BeadChip array across the TEAD3 gene region. Upper panel: UCSC genome browser shot detailing location of Illumina CpG probes, histone marks predictive of enhancers, CpG island(s) and conserved transcription factor binding sites (TFBS) associated with the gene body. Lower panel: DNA methylation pattern at Illumina CpG sites across the TEA domain family member 3 (TEAD3) gene region in control (n = 19) and early onset pre-eclampsia (EOPET; n = 19) placentae. The Illumina CpG probe identifier is indicated by cg# and the position of CpG sites on the graphs are not to scale. Values are mean ± SD, and *P<0.05 based on Mann-Whitney tests.

(DOCX)

Figure S6 CpG sites on the Illumina Infinium Human-Methylation450 BeadChip array across the CYP19 gene region. Upper panel: UCSC genome browser shot detailing location of Illumina CpG probes, histone marks predictive of enhancers, CpG island(s) and conserved transcription factor binding sites (TFBS) associated with the gene body. Lower panel: DNA methylation pattern at Illumina CpG sites across the CYP19 gene region in control (n = 19) and early onset pre-eclampsia (EOPET; n = 19) placentae. The Illumina CpG probe identifier is indicated by cg# and the position of CpG sites on the graphs are not to scale. Values are mean ± SD, and *P<0.05 based on Mann-Whitney tests.

(DOCX)

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