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Maternal weight-related variables are associated with placental DNA methylation status at selected glucocorticoid-pathway loci in term pregnancies

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Abstract The placenta regulates foetal exposure to glucocorticoids through local cortisol metabolism and glucocorticoid receptor signalling. Key components of this pathway include 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) and type 2 (11 β -HSD2), and the glucocorticoid receptor (GR). This study investigated whether maternal characteristics and perinatal factors are associated with placental DNA methylation status at selected glucocorticoid-pathway loci, including *HSD11B1*, *HSD11B2*, and *NR3C1*, in physiological term pregnancies. Placental samples were obtained from 70 term singleton pregnancies. DNA methylation was quantified by methylation-specific qPCR and expressed as a demethylation index (DMI). Delivery mode was not associated with placental DMI of *HSD11B1*, *HSD11B2* island 1 and island 2, or *NR3C1*. Instead, several nominal gene- and locus-specific associations were observed with maternal weight-related variables. *HSD11B1* DMI showed a nominal positive association with gestational weight gain, whereas *HSD11B2* DMI at both CpG islands showed distinct nominal associations with pre-pregnancy BMI and gestational weight gain. *NR3C1* DMI was nominally associated with BMI at delivery only in vaginal deliveries. Delivery mode was not associated with DMI markers at the analysed glucocorticoid-pathway loci. Maternal weight-related variables showed nominal associations with *HSD11B1* and *HSD11B2* DMI, suggesting that maternal metabolic status may be relevant to placental epigenetic variation. *HSD11B1* DMI was independently

associated with placental 11 β -HSD2 activity, whereas no DMI marker was independently associated with cord cortisol.

Keywords: DNA methylation; placenta; gestational weight gain; *HSD11B1*; *HSD11B2*; *NR3C1*

1 Introduction

Glucocorticoids are steroid hormones secreted by the adrenal cortex that regulate carbohydrate, protein, and lipid metabolism, modulate immune responses, contribute to water-electrolyte balance, and blood pressure regulation, and influence bone metabolism, pain perception, and neuroendocrine function. The main endogenous glucocorticoid in humans is cortisol. Cortisol secretion is regulated through negative feedback within the hypothalamic-pituitary-adrenal (HPA) axis, whereas intracellular glucocorticoid availability is modulated by two 11 β -hydroxysteroid dehydrogenase enzymes, 11 β -HSD1 and 11 β -HSD2, encoded by *HSD11B1* (1q32.2) and *HSD11B2* (16q22.1) genes, respectively. Because cortisol is lipophilic, it diffuses across the cell membrane and binds to the cytoplasmic glucocorticoid receptor, encoded by *NR3C1* (5q31.3). Upon ligand binding, GR translocates from the cytoplasm to the nucleus, where it acts as both a transcriptional activator and repressor. The activated GR complex enhances the expression of anti-inflammatory proteins and inhibits the expression of pro-inflammatory mediators, in part by interfering with the nuclear translocation or activity of other transcription factors [1,2]. Thus, GR plays an important role in inflammatory responses, cellular proliferation, and differentiation in target tissues.

Glucocorticoids are essential for normal pregnancy, including implantation, placental development, foetal growth, maturation, and parturition. Although foetal exposure to cortisol is required for maturation, prolonged or excessive exposure has been associated with lower birth weight and increased risk of adult-onset diseases such as hypertension, diabetes mellitus, and stroke [3–5]. During pregnancy, the foetus is partly protected from excessive maternal cortisol exposure by placental 11 β -HSD2, which

converts active cortisol to inactive cortisone. In contrast, 11 β -HSD1 catalyses the reverse reaction in vivo, regenerating cortisol from cortisone. Placental 11 β -HSD2 therefore functions as a glucocorticoid barrier, with activity increasing during gestation and reaching high levels in the third trimester [6,7].

HSD11B2 is also expressed in the foetal brain during the second trimester, suggesting a potential neuroprotective role [8,9]. Excessive foetal cortisol exposure has been implicated in altered foetal growth and later may contribute to cardiovascular disease, type 2 diabetes [10], psychiatric disorders [11], and impaired postnatal brain function [12]. Therefore, tight regulation of glucocorticoid levels is a critical determinant of foetal well-being both prenatally and postnatally. Maternal stress generally reduces placental 11 β -HSD2 activity [13–15] although some studies have found the opposite pattern [13]. These observations highlight the need to examine placental glucocorticoid regulation in relation to specific maternal and perinatal factors.

The placenta connects the developing foetus with the maternal circulation and supports nutrient transfer, gas exchange, hormone production, pregnancy maintenance, and labour. Proper implantation and placental development are essential for optimal foetal growth and well-being. Epigenetic modifications, including DNA methylation in placental tissue, have been implicated in the regulation of foetal development and may predispose the offspring to diseases in later life. DNA methylation at CpG islands within gene promoter regions is often associated with suppression or silencing of gene expression [16,17]. Altered *NR3C1* expression during prenatal development has been associated with child neurobehavioral and mental health disorders [18], whereas aberrant *HSD11B2* expression has been linked to foetal growth restriction and low birth weight [19].

This study investigated whether maternal characteristics and perinatal factors are associated with placental DNA methylation status at selected regulatory regions of *HSD11B1*, *HSD11B2*, and *NR3C1* in physiological term pregnancies. We focused on gestational weight gain, delivery mode, and labour analgesia because these factors may reflect maternal metabolic status and perinatal stress-related conditions relevant to placental glucocorticoid regulation. We treated DNA methylation as a potential regulatory

marker and hypothesized that maternal and perinatal factors would be associated with variation in placental demethylation markers status at the analysed loci.

2 Results

2.1 Characteristics of the study group

A total of 70 Caucasian women with singleton pregnancies and gestational age at least 37 weeks were divided into two groups. The caesarean section group included 43 women and vaginal delivery group included 27 women. The clinical characteristics of the study group are summarized in Table 1. No significant differences were observed between the caesarean section and vaginal delivery groups for the analysed clinical variables (all $P > 0.05$).

Table 1. Clinical characteristics of the study group.

Variable, parameter	Unit or category	Total group (N=70)	Caesarean section (N=43)	Vaginal delivery (N=27)	P^1	FDR ²
Maternal age, Me (IQR)	years	32 (28–35)	33 (28–37)	31 (28–33)	0.143	0.524
Gestational age (weeks), n (%)	37	6 (8.57)	3 (6.98)	3 (11.11)	0.067	0.369
	38	11 (15.71)	7 (16.28)	4 (14.81)		
	39	32 (45.71)	24 (55.82)	8 (29.63)		
	40	9 (12.86)	2 (4.65)	7 (25.93)		
	41	12 (17.14)	7 (16.28)	5 (18.52)		
Parity, n (%)	1	38 (54.29)	24 (55.81)	14 (51.85)	0.601	0.944
	2	29 (41.43)	18 (41.86)	11 (40.74)		
	3	3 (4.29)	1 (2.33)	2 (7.41)		
Pre-pregnancy BMI, Me (IQR)	kg/m ²	22.89 (20.82–25.01)	22.92 (19.47–25.46)	22.86 (21.26–24.91)	0.990	0.990
BMI at delivery, Me (IQR)	kg/m ²	28.49 (25.95–30.80)	28.70 (25.71–30.85)	28.26 (26.64–30.45)	0.842	0.990
Gestational weight gain, Me (IQR)	kg	16 (11–19)	16 (12–20)	15 (11–18)	0.463	0.931
	%	25.00 (18.33–31.75)	26.67 (19.30–32.56)	22.22 (16.92–29.23)	0.402	0.931

Newborn weight, Me (IQR)	kg	3.58 (3.30–3.85)	3.55 (3.34–3.85)	3.65 (3.30–3.90)	0.966	0.990
Newborn sex, n (%)	male	32 (45.71)	21 (48.84)	11 (40.74)	0.508	0.931
	female	38 (54.29)	22 (51.16)	16 (59.26)		
Apgar score at 1 st minute, n (%)	8	2 (2.86)	2 (4.65)	0 (0.00)	0.059	0.369
	9	10 (14.29)	9 (20.93)	1 (3.70)		
	10	58 (82.86)	32 (74.42)	26 (96.30)		
Apgar score at 5 th minute, n (%)	9	3 (4.29)	2 (4.65)	1 (3.70)	0.849	0.990
	10	67 (95.71)	41 (95.35)	26 (96.30)		
Duration of uterine contraction ³ , Me (IQR)	hours	-	-	5.17 (3.42–7.67)	-	-
Analgesia ⁴ , n (%)	yes	59 (84.29)	43 (100.00)	16 (59.26)	-	-

Me – median; IQR – interquartile range (25th–75th percentile).

¹Mann-Whitney U test or chi-square test were used to compare continuous or categorical variables, respectively, between delivery-mode groups.

² Benjamini–Hochberg false discovery rate (FDR) correction; repeated P-values in multi-row categorical variables were counted once per tested variable; number of P-values in a family was 11.

³ Only in vaginal deliveries.

⁴ Refers to spinal analgesia for caesarean section or epidural analgesia for vaginal delivery.

2.2 *HSD11B1* methylation status in placenta

The *HSD11B1* gene contains no predicted CpG island; therefore, methylation was assessed within a 203-bp fragment located upstream of the transcription start site (Figure 1). This amplicon includes three CpG dinucleotides within a sequence showing promoter-like signatures for *HSD11B1* transcript variant 1 (E1418619/prom, UCSC Genome Browser). Selection of these CpG sites was based on previous studies of human placenta [20]. Placental *HSD11B1* DMI did not differ significantly between the caesarean section and vaginal delivery groups ($P = 0.297$; Figure 1).

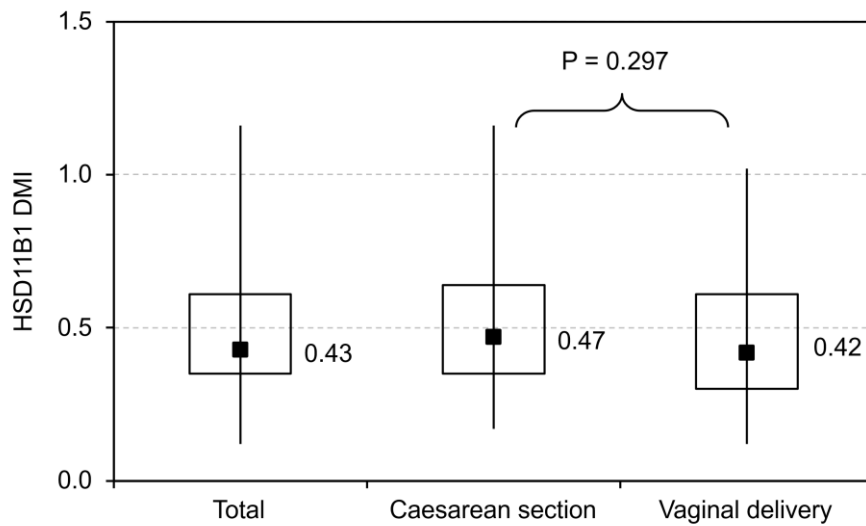


Fig. 1. *HSD11B1* DMI by delivery mode. *HSD11B1* demethylation index (DMI) in the total group and according to delivery mode. P value was calculated using the Mann-Whitney U test. Midpoint, median; box, interquartile range (25th–75th percentile); whiskers, minimum–maximum.

HSD11B1 DMI was not correlated with maternal age, gestational age, parity, newborn weight, newborn ponderal index, or Apgar scores in the total group or in delivery mode subgroups (Table 2). In the total group, *HSD11B1* DMI showed nominal correlations with gestational weight gain expressed both in kilograms ($r = 0.290$, $P = 0.015$) and as percentage ($r = 0.236$, $P = 0.049$). In vaginal deliveries, *HSD11B1* DMI showed nominal negative correlation with pre-pregnancy BMI ($r = -0.414$, $P =$

0.032) and positive with gestational weight gain (%) ($r = 0.411$, $P = 0.033$) and duration of uterine contractions ($r = 0.492$, $P = 0.009$). *HSD11B1* DMI was also nominally higher in placentas from female than male newborns in the total group (0.47 vs. 0.37, $P = 0.018$, Figure 2). In vaginal deliveries, *HSD11B1* DMI was nominally higher in placentas from women who received epidural analgesia than in those without epidural analgesia (0.54 vs 0.36, $P = 0.032$, Figure 3). None of these associations remained significant after FDR correction. Within the epidural analgesia subgroup, *HSD11B1* DMI did not differ by newborn sex.

In summary, *HSD11B1* DMI was not associated with delivery mode, but showed modest, nominal associations with gestational weight gain and selected subgroup specific variables, including pre-pregnancy BMI, newborn sex, epidural analgesia and duration of uterine contractions.

Table 2. Correlations between *HSD11B2* island 1 demethylation index (DMI) and maternal or offspring outcomes.

Variable	Method ¹	Total group (N=70)			Caesarean section (N=43)			Vaginal delivery (N=27)		
		Test	<i>P</i>	FDR ²	Test	<i>P</i>	FDR ²	Test	<i>P</i>	FDR ²
Maternal age (years)	r	0.007	0.954	0.974	−0.034	0.828	0.903	−0.030	0.882	0.931
Gestational age (weeks)	r	0.004	0.974	0.974	−0.078	0.619	0.832	0.061	0.763	0.931
Parity	r	0.198	0.100	0.300	0.243	0.117	0.351	0.175	0.382	0.764
Pre-pregnancy BMI (kg/m ²)	r	0.301	0.011	0.066	0.381	0.012	0.072	0.184	0.360	0.764
BMI at delivery (kg/m ²)	r	0.177	0.142	0.336	0.279	0.070	0.280	0.020	0.921	0.931

Gestational weight gain (kg)	r	-0.215	0.073	0.292	-0.204	0.189	0.454	-0.336	0.086	0.401
Gestational weight gain (%)	r	-0.319	0.007	0.066	-0.382	0.012	0.072	-0.366	0.060	0.401
Newborn weight (kg)	r	0.106	0.383	0.657	0.148	0.342	0.684	0.017	0.931	0.931
Newborn ponderal index	r	0.167	0.168	0.336	-0.077	0.624	0.832	0.450	0.019	0.266
Newborn sex	Z	0.660	0.509	0.691	0.802	0.423	0.725	-0.222	0.824	0.931
Apgar score at 1 st minute	r	-0.007	0.953	0.974	0.010	0.949	0.949	0.050	0.803	0.931
Apgar score at 5 th minute	r	0.079	0.518	0.691	0.044	0.777	0.903	0.050	0.803	0.931
Epidural analgesia ³	Z	—	—	—	—	—	—	-0.913	0.361	0.764
Duration of uterine contraction (hours) ³	r	—	—	—	—	—	—	-0.206	0.302	0.764

¹ r – Spearman correlation coefficient; Z – Mann-Whitney U test.

² Benjamini–Hochberg false discovery rate (FDR) correction; FDR families: total group, caesarean section, and vaginal delivery were corrected separately. The number of *P*-values in a family was 12 for the total group and caesarean subgroup and 14 for the vaginal delivery subgroup.

³ Only in vaginal deliveries.

Nominally significant correlations ($P < 0.05$) are in bold; none remained significant after FDR correction.

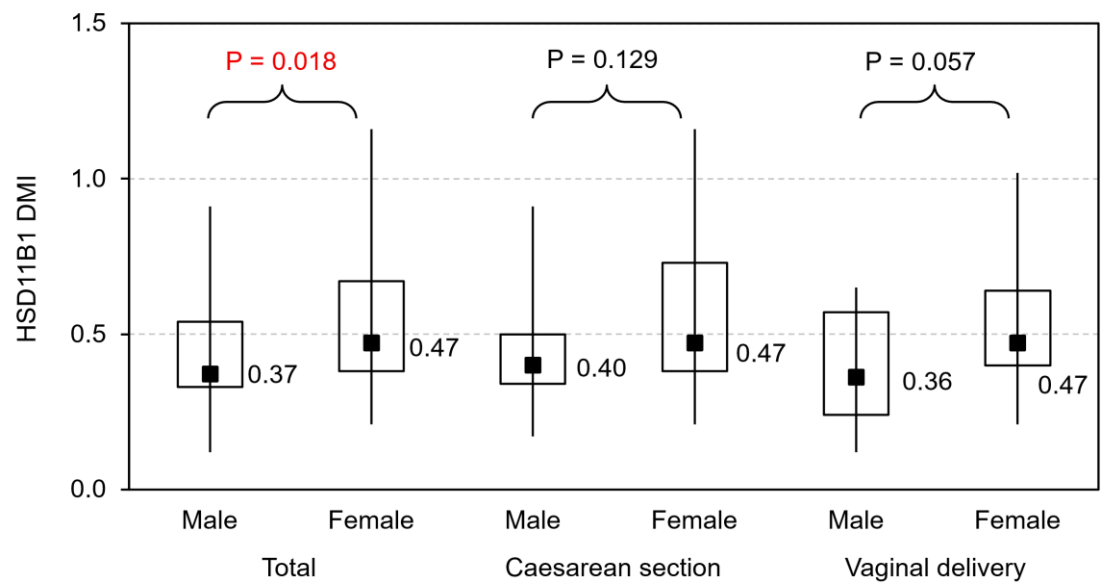


Fig. 2. *HSD11B1* DMI by newborn sex. *HSD11B1* demethylation index (DMI) according to newborn sex in the total group and according to delivery mode. P values were calculated using the Mann-Whitney U test. Midpoint, median; box, interquartile range (25th–75th percentile); whiskers, minimum–maximum.

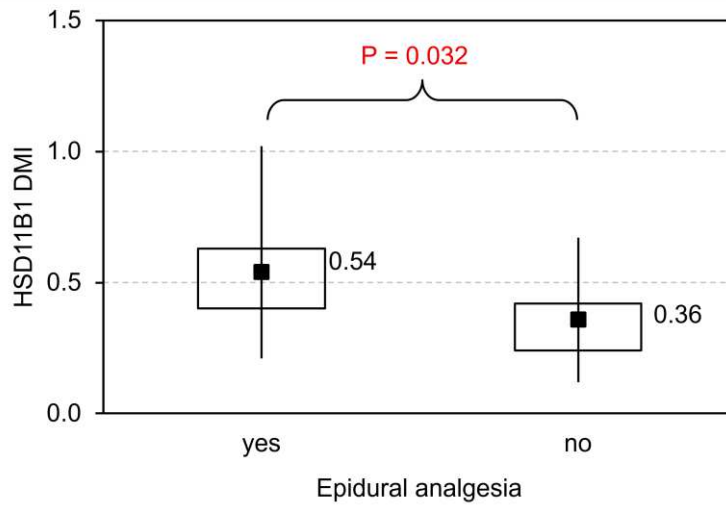


Fig. 3. *HSD11B1* DMI by epidural analgesia. *HSD11B1* demethylation index (DMI) according to epidural analgesia status in vaginal deliveries. *P* value was calculated using the Mann-Whitney U test. Midpoint, median; box, interquartile range (25th–75th percentile); whiskers, minimum–maximum.

2.3 *HSD11B2* methylation status in placenta

Analysis of the *HSD11B2* sequence identified two CpG islands: island 1, located from –758 to –226 bp upstream of the translation start site, and island 2, located from –204 bp to +330 bp and spanning exon 1. Methylation was assessed within a 122-bp fragment in *HSD11B2* island 1 and a 170-bp fragment in *HSD11B2* island 2. These amplicons contained 12 and 20 CpG dinucleotides, respectively (Supplementary Table S1). Placental *HSD11B2* island 1 DMI did not differ significantly between the caesarean section and vaginal delivery groups ($P = 0.138$; Figure 4A). Similarly, *HSD11B2* island 2 DMI did not differ significantly between delivery-mode groups ($P = 0.952$; Figure 4B). DMI values for *HSD11B2* island 1 and *HSD11B2* island 2 were not significantly correlated with each other in the total group ($r = 0.109$, $P = 0.368$), caesarean section group ($r = 0.209$, $P = 0.178$), or vaginal delivery group ($r = -0.025$, $P = 0.901$).

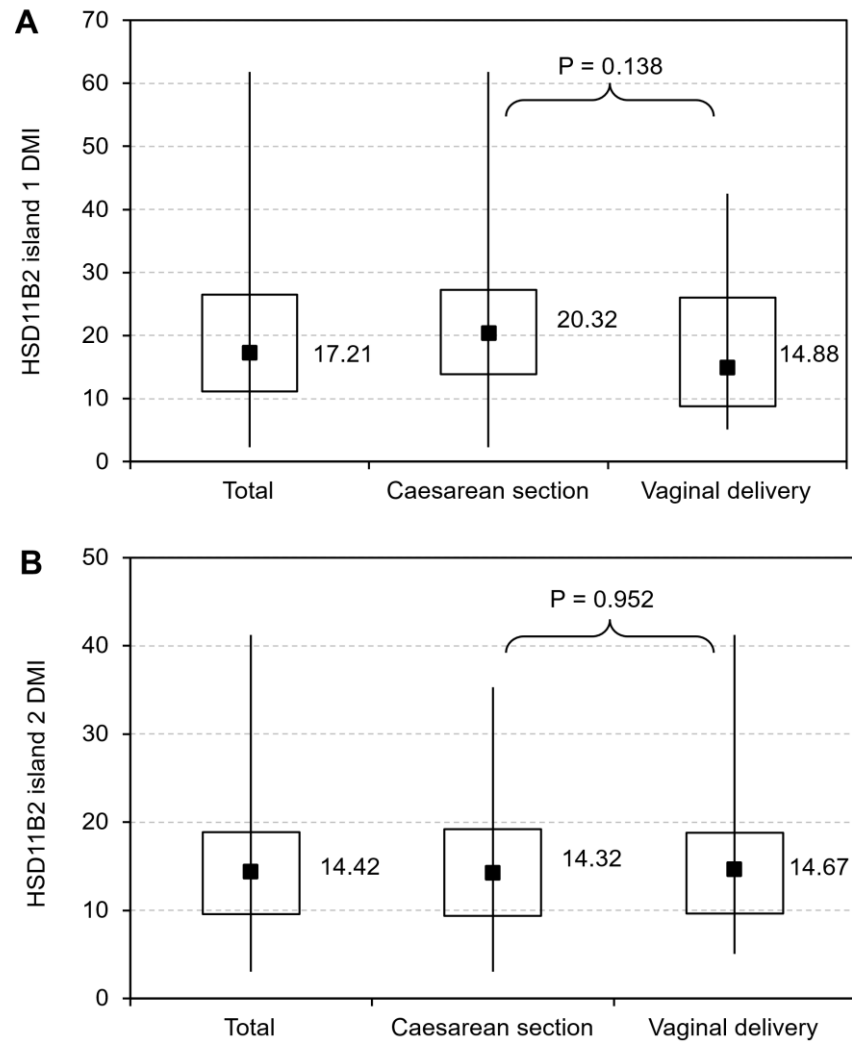


Fig. 4. *HSD11B2* DMI by delivery mode. *HSD11B2* demethylation index (DMI) in the total group and according to delivery mode. (A) *HSD11B2* island 1 DMI. (B) *HSD11B2* island 2 DMI. P values were calculated using the Mann-Whitney U test. Midpoint, median; box, interquartile range (25th–75th percentile); whiskers, minimum–maximum.

For *HSD11B2* island 1, DMI was positively correlated with pre-pregnancy BMI in the total group ($r = 0.301$, $P = 0.011$) and in the caesarean section group ($r = 0.381$, $P = 0.012$), but not in the vaginal delivery group. *HSD11B2* island 1 DMI was negatively correlated with gestational weight gain (%) in the total group ($r = -0.319$, $P = 0.007$) and in the caesarean section group ($r = -0.382$, $P = 0.012$), with a near-significant negative correlation in vaginal deliveries ($r = -0.366$, $P = 0.060$). In vaginal deliveries, *HSD11B2* island 1 DMI was also positively correlated with newborn ponderal index ($r = 0.450$, $P = 0.019$). No other tested maternal, perinatal, or newborn variables were significantly correlated with *HSD11B2* island 1 DMI (Table 3). For *HSD11B2* island 2, DMI was positively correlated with gestational weight gain expressed in kilograms in the total group ($r = 0.257$, $P = 0.032$) and in the caesarean section group ($r = 0.308$, $P = 0.045$), but not in the vaginal delivery group. In vaginal deliveries, *HSD11B2* island 2 DMI was negatively correlated with gestational age ($r = -0.426$, $P = 0.027$). No other tested maternal, perinatal, or newborn variables were significantly correlated with *HSD11B2* island 2 DMI (Table 4). These correlations were nominal and did not remain significant after FDR correction.

Table 3. Correlations between *HSD11B2* island 1 demethylation index (DMI) and maternal or offspring outcomes.

Variable	Method ¹	Total group (N=70)			Caesarean section (N=43)			Vaginal delivery (N=27)		
		Test	<i>P</i>	FDR ²	Test	<i>P</i>	FDR ²	Test	<i>P</i>	FDR ²
Maternal age (years)	r	0.007	0.954	0.974	-0.034	0.828	0.903	-0.030	0.882	0.931
Gestational age (weeks)	r	0.004	0.974	0.974	-0.078	0.619	0.832	0.061	0.763	0.931
Parity	r	0.198	0.100	0.300	0.243	0.117	0.351	0.175	0.382	0.764
Pre-pregnancy BMI (kg/m ²)	r	0.301	0.011	0.066	0.381	0.012	0.072	0.184	0.360	0.764
BMI at delivery (kg/m ²)	r	0.177	0.142	0.336	0.279	0.070	0.280	0.020	0.921	0.931

Gestational weight gain (kg)	r	-0.215	0.073	0.292	-0.204	0.189	0.454	-0.336	0.086	0.401
Gestational weight gain (%)	r	-0.319	0.007	0.066	-0.382	0.012	0.072	-0.366	0.060	0.401
Newborn weight (kg)	r	0.106	0.383	0.657	0.148	0.342	0.684	0.017	0.931	0.931
Newborn ponderal index	r	0.167	0.168	0.336	-0.077	0.624	0.832	0.450	0.019	0.266
Newborn sex	Z	0.660	0.509	0.691	0.802	0.423	0.725	-0.22 2	0.824	0.931
Apgar score at 1 st minute	r	-0.007	0.953	0.974	0.010	0.949	0.949	0.050	0.803	0.931
Apgar score at 5 th minute	r	0.079	0.518	0.691	0.044	0.777	0.903	0.050	0.803	0.931
Epidural analgesia ₃	Z	–	–	–	–	–	–	-0.91 3	0.361	0.764
Duration of uterine contraction (hours) ³	r	–	–	–	–	–	–	-0.206	0.302	0.764

¹ r – Spearman correlation coefficient; Z – Mann-Whitney U test.

² Benjamini–Hochberg false discovery rate (FDR) correction; FDR families: total group, caesarean section, and vaginal delivery were corrected separately. The number of *P*-values in a family was 12 for the total group and caesarean subgroup and 14 for the vaginal delivery subgroup.

³ Only in vaginal deliveries.

Nominally significant correlations ($P < 0.05$) are in bold; none remained significant after FDR correction

Table 4. Correlations between *HSD11B2* island 2 demethylation index (DMI) and maternal or offspring outcomes.

Variable	Method ¹	Total group (N=70)			Caesarean section (N=43)			Vaginal delivery (N=27)		
		Test	<i>P</i>	FDR ²	Test	<i>P</i>	FDR ²	Test	<i>P</i>	FDR ²
Maternal age (years)	r	−0.064	0.599	0.653	−0.137	0.380	0.570	0.056	0.780	1.000
Gestational age (weeks)	r	−0.105	0.388	0.619	0.119	0.448	0.593	−0.426	0.027	0.378
Parity	r	−0.084	0.487	0.649	−0.031	0.845	0.922	−0.175	0.381	1.000
Pre-pregnancy BMI (kg/m ²)	r	−0.030	0.806	0.806	0.013	0.935	0.935	−0.064	0.752	1.000
BMI at delivery (kg/m ²)	r	0.108	0.373	0.619	0.148	0.343	0.570	0.015	0.940	1.000
Gestational weight gain (kg)	r	0.257	0.032	0.384	0.308	0.045	0.405	0.150	0.455	1.000
Gestational weight gain (%)	r	0.177	0.142	0.538	0.194	0.213	0.511	0.155	0.439	1.000
Newborn weight (kg)	r	0.147	0.224	0.538	0.260	0.093	0.405	−0.055	0.785	1.000
Newborn ponderal index	r	0.170	0.159	0.538	0.253	0.102	0.405	0.021	0.917	1.000
Newborn sex	Z	1.261	0.207	0.538	1.494	0.135	0.405	0.222	0.824	1.000
Apgar score at 1 st minute	r	−0.067	0.584	0.653	−0.107	0.494	0.593	0.000	1.000	1.000

Apgar score at 5 th minute	r	0.099	0.413	0.619	0.169	0.278	0.556	0.000	1.000	1.000
Epidural analgesia ³	Z	–	–	–	–	–	–	–0.469	0.639	1.000
Duration of uterine contraction (hours) ³	r	–	–	–	–	–	–	0.286	0.148	1.000

¹ r – Spearman correlation coefficient; Z – Mann-Whitney U test.

² Benjamini–Hochberg false discovery rate (FDR) correction; FDR families: total group, caesarean section, and vaginal delivery were corrected separately. The number of *P*-values in a family was 12 for the total group and caesarean subgroup and 14 for the vaginal delivery subgroup.

³ Only in vaginal deliveries.

Nominally significant correlations ($P < 0.05$) are in bold; none remained significant after FDR correction

In summary, *HSD11B2* DMI was not associated with delivery mode. *HSD11B2* island 1 and island 2 showed distinct, non-overlapping nominal correlation patterns, mainly involving maternal weight-related variables; however, none of these associations remained significant after FDR correction.

2.4 *NR3C1* methylation status in placenta

For *NR3C1*, methylation was analysed within the large CpG island located in the proximal promoter region. This CpG island spans seven non-coding first exons, 1B–1F, 1H, and 1J, which act as of alternative promoters [21]. Based on previous reports [22], we selected the exon 1F promoter region and assessed methylation within a 138-bp fragment containing 12 CpG dinucleotides (Supplementary Table S1).

NR3C1 DMI did not differ significantly between vaginal deliveries and caesarean sections ($P = 0.344$, Figure 5). *NR3C1* DMI showed nominal positive correlation with BMI at delivery ($r = 0.434$, $P = 0.024$) and negative correlation with newborn ponderal index ($r = -0.510$, $P = 0.007$) in the vaginal delivery group, but not in the total group or caesarean section group; however, these associations did not remain significant after FDR correction. No significant correlations were observed with other maternal, perinatal, or newborn variables in the total group (Table 5).

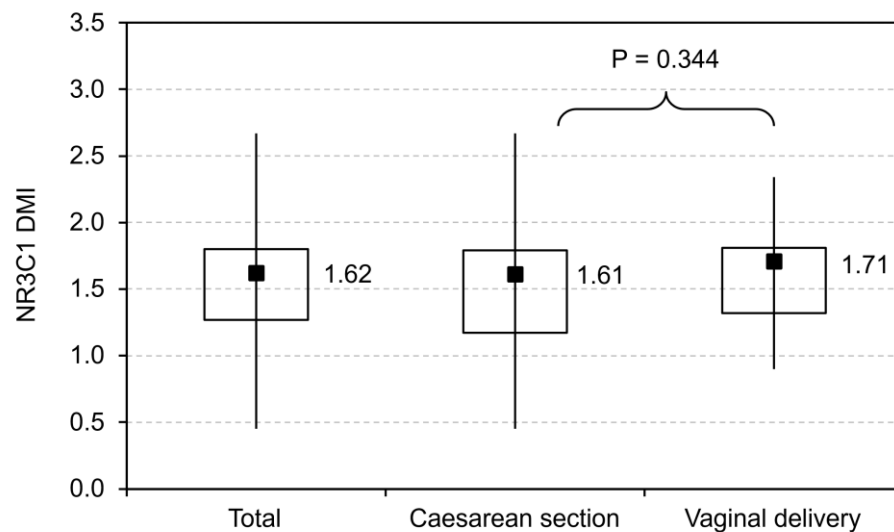


Fig. 5. *NR3C1* DMI by delivery mode. *NR3C1* demethylation index (DMI) in the total group and according to delivery mode. P values were calculated using the Mann-Whitney U test. Midpoint, median; box, interquartile range (25th–75th percentile); whiskers, minimum–maximum.

In summary, *NR3C1* DMI was not associated with delivery mode but showed nominal vaginal delivery-specific associations with BMI at delivery and newborn ponderal index.

Table 5. Correlations between *NR3C1* demethylation index (DMI) and maternal or offspring outcomes

Variable	Method ¹	Total group (N=70)			Caesarean section (N=43)			Vaginal delivery (N=27)		
		Test	<i>P</i>	FDR ²	Test	<i>P</i>	FDR ²	Test	<i>P</i>	FDR ²
Maternal age (years)	r	−0.106	0.381	0.637	−0.067	0.668	0.996	−0.100	0.618	0.767
Gestational age (weeks)	r	−0.099	0.415	0.637	0.049	0.753	0.996	−0.350	0.074	0.259
Parity	r	−0.052	0.671	0.732	−0.190	0.221	0.884	0.108	0.591	0.767
Pre-pregnancy BMI (kg/m ²)	r	0.079	0.517	0.689	0.001	0.996	0.996	0.234	0.241	0.422
BMI at delivery (kg/m ²)	r	0.143	0.237	0.583	−0.013	0.935	0.996	0.434	0.024	0.168
Gestational weight gain (kg)	r	0.141	0.243	0.583	0.040	0.801	0.996	0.371	0.057	0.259
Gestational weight gain (%)	r	0.097	0.425	0.637	0.022	0.890	0.996	0.326	0.097	0.272
Newborn weight (kg)	r	−0.029	0.813	0.813	0.003	0.985	0.996	−0.006	0.975	0.980
Newborn ponderal index	r	−0.170	0.159	0.583	0.089	0.569	0.996	−0.510	0.007	0.098

Newborn sex	Z	1.421	0.155	0.583	1.919	0.055	0.660	0.025	0.980	0.980
Apgar score at 1 st minute	r	0.162	0.181	0.583	0.237	0.127	0.762	−0.252	0.205	0.410
Apgar score at 5 th minute	r	−0.052	0.667	0.732	0.049	0.755	0.996	−0.252	0.205	0.410
Epidural analgesia ³	Z	-	-	-	-	-	-	0.441	0.657	0.767
Duration of uterine contraction (hours) ³	r	-	-	-	-	-	-	0.213	0.287	0.446

¹r – Spearman correlation coefficient; Z – Mann-Whitney U test.

² Benjamini–Hochberg false discovery rate (FDR) correction; FDR families: total group, caesarean section, and vaginal delivery were corrected separately. The number of *P*-values in a family was 12 for the total group and caesarean subgroup and 14 for the vaginal delivery subgroup.

³ Only in vaginal deliveries.

Nominally significant correlations ($P < 0.05$) are in bold; none remained significant after FDR correction.

2.5 Exploratory associations of cord cortisol and placental 11 β -HSD2 activity with clinical variables and gene-specific DMI markers

Cord cortisol concentration and placental 11 β -HSD2 activity were analysed as primary outcomes in relation to gene-specific DMI markers. Cord cortisol was significantly higher in vaginal deliveries than in caesarean sections ($P = 0.001$) (Figure 6A), whereas placental 11 β -HSD2 activity did not differ significantly between delivery-mode groups (Figure 6B). In addition, cord cortisol remained negatively correlated with placental 11 β -HSD2 activity in vaginal deliveries ($r = -0.487$, $P = 0.010$, FDR = 0.030) (Table 6).

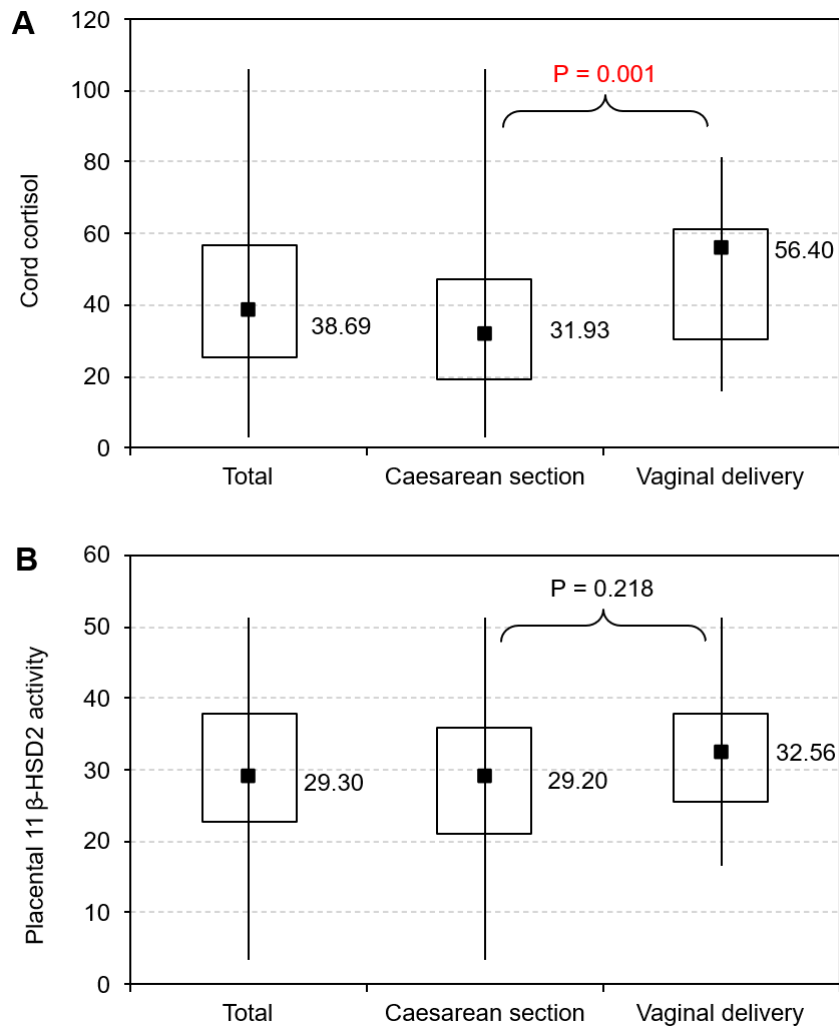


Fig. 6. Cord cortisol and placental 11 β -HSD2 activity by delivery mode. (A) Cord cortisol concentration ($\mu\text{g/dL}$) and (B) placental 11 β -HSD2 activity (pg cortisone/mg protein/min) in the total group and according to delivery mode. P values were calculated using the Mann-Whitney U test. Midpoint, median; box, interquartile range (25th–75th percentile); whiskers, minimum–maximum.

Exploratory unadjusted analyses using Spearman correlations were first performed to identify clinical variables associated with these two primary outcomes. In the total group, cord cortisol was positively correlated with gestational weight gain (%) ($r = 0.281$, $P = 0.018$) and negatively correlated with newborn ponderal index ($r = -0.272$,

$P = 0.023$). In vaginal deliveries, cord cortisol was positively correlated with labour duration ($r = 0.387$, $P = 0.046$). Placental 11 β -HSD2 activity was negatively correlated with gestational weight gain both in kilograms ($r = -0.254$, $P = 0.034$) and as percentage ($r = -0.237$, $P = 0.048$), and positively correlated with gestational age ($r = 0.247$, $P = 0.039$) and cord pH ($r = 0.329$, $P = 0.013$). These clinical correlations were nominal and did not remain significant after FDR correction (Supplementary Table S3).

Unadjusted correlations between the primary outcomes and gene-specific DMI markers are presented in Table 6. After FDR correction, cord cortisol remained negatively correlated with *HSD11B2* island 1 DMI in the total group ($r = -0.444$, $P = 0.001$, FDR = 0.004) and in vaginal deliveries ($r = -0.513$, $P = 0.006$, FDR = 0.024). Placental 11 β -HSD2 activity remained negatively correlated with *HSD11B1* DMI in the total group ($r = -0.423$, $P = 0.001$, FDR = 0.004) and in the caesarean section subgroup ($r = -0.467$, $P = 0.002$, FDR = 0.008). Other associations reached nominal significance only and should be interpreted as exploratory and unadjusted.

Table 6. Unadjusted Spearman correlations of cord cortisol and placental 11 β -HSD2 activity with gene-specific demethylation index (DMI) markers

	Total group (N=70)			Caesarean section (N=43)			Vaginal delivery (N=27)		
Predictor	r	P	FDR ¹	r	P	FDR ¹	r	P	FDR ¹
	Outcome: cord cortisol								
DMI NR3C1	0.240	0.046	0.092	0.139	0.373	0.468	0.373	0.055	0.110
DMI HSD11B1	-0.038	0.754	0.957	-0.124	0.428	0.468	0.201	0.315	0.315
DMI HSD11B2 island 1	-0.444	0.001	0.004	-0.318	0.038	0.152	-0.513	0.006	0.024

DMI HSD11B2 island 2	0.007	0.957	0.957	−0.114	0.468	0.468	0.271	0.171	0.228
	Outcome: Placental 11β-HSD2 activity								
DMI NR3C1	0.010	0.934	0.934	0.119	0.448	0.597	−0.149	0.459	0.494
DMI HSD11B1	−0.423	0.001	0.004	−0.467	0.002	0.008	−0.287	0.147	0.494
DMI HSD11B2 island 1	−0.024	0.843	0.934	0.074	0.638	0.638	−0.137	0.494	0.494
DMI HSD11B2 island 2	−0.240	0.046	0.092	−0.272	0.078	0.156	−0.198	0.321	0.494
	Outcome: cord cortisol								
Placental 11 β -HSD2 activity	−0.0003	0.998	0.998	0.127	0.418	0.627	−0.487	0.010	0.030

r – Spearman's coefficient.

¹ Benjamini–Hochberg false discovery rate (FDR) correction. For gene-specific DMI correlations, total group, caesarean section, and vaginal delivery were corrected separately for each outcome, with four *P*-values per family. The correlation between placental 11 β -HSD2 activity and cord cortisol was corrected separately across the three analysis groups.

Nominal *P*-values < 0.05 and FDR < 0.05 are in bold.

2.6 Adjusted multivariable regression models for cord cortisol and placental 11 β -HSD2 activity

Multivariable linear regression models were fitted to assess whether selected DMI markers remained independently associated with cord cortisol and placental 11 β -HSD2 activity after adjustment for gestational age, gestational weight gain (%), and delivery mode.

For cord cortisol, all three adjusted models were significant overall (Table 7). However, none of the tested DMI markers remained independently associated with cord cortisol after adjustment, including *HSD11B2* island 1 DMI, *NR3C1* DMI, and *HSD11B1* DMI. Delivery mode remained independently associated with cord cortisol in all three models, whereas gestational weight gain (%) remained significant in models C2 and C3 (Supplementary Table S4).

For placental 11 β -HSD2 activity, the model including *HSD11B2* island 2 DMI was significant overall, but *HSD11B2* island 2 DMI itself did not remain independently associated with the outcome after adjustment. In contrast, *HSD11B1* DMI remained independently associated with lower placental 11 β -HSD2 activity in model P2 ($B = -10.831$, $\beta = -0.290$, $P = 0.017$). The model including *HSD11B2* island 1 DMI was not significant overall, and *HSD11B2* island 1 DMI was not associated with placental 11 β -HSD2 activity.

Overall, several unadjusted associations, some of which remained significant after FDR correction, were observed before covariate adjustment. After covariate adjustment, however, only *HSD11B1* DMI remained independently associated with placental 11 β -HSD2 activity, whereas no DMI marker remained independently associated with cord cortisol.

Table 7. Adjusted multivariable linear regression models for cord cortisol and placental 11 β -HSD2 activity. Overall model fit and independent association of the primary gene-specific demethylation index (DMI) predictors are shown. Full coefficients for all adjusted models are provided in Supplementary Table S4.

Model	Model fit				Gene-specific predictor				
	R ²	Adjusted R ²	Model <i>P</i>	N	Main predictor	B	SE	Standardized β	<i>P</i>
Outcome: cord cortisol									
C1	0.253	0.207	0.001	70	DMI <i>HSD11B2</i> island 1	-0.333	0.203	-0.200	0.105

C2	0.250	0.204	0.001	70	DMI NR3C1		8.051	5.111	0.170	0.120
C3	0.237	0.190	0.001	70	DMI HSD11B1		-9.891	8.576	-0.132	0.253
Outcome: placenta 11β-HSD2 activity										
P1	0.138	0.084	0.045	70	DMI HSD11B2 is- land 2		-0.244	0.154	-0.185	0.117
P2	0.180	0.129	0.011	70	DMI HSD11B1		-10.831	4.420	-0.290	0.017
P3	0.106	0.051	0.117	70	DMI HSD11B2 is- land 1		-0.038	0.110	-0.047	0.728

Table 8. All models were adjusted for gestational age (weeks), gestational weight gain (%), and delivery mode. B, unstandardized regression coefficient; SE, standard error; β , standardized regression coefficient. Significant *P*-values are in bold.

3 Discussion

In this study of term singleton pregnancies, we investigated placental DMI markers at selected glucocorticoid-pathway loci in relation to delivery mode, maternal and newborn characteristics, cord cortisol, and placental 11 β -HSD2 activity. The main finding was that delivery mode was not associated with placental DMI of *HSD11B1*, *HSD11B2* (island 1 and island 2), or *NR3C1*. Instead, several nominal gene- and locus-specific associations were observed with maternal weight-related variables. Although unadjusted analyses suggested associations of selected DMI markers with cord cortisol and placental 11 β -HSD2 activity, most of these associations did not remain significant after adjustment for gestational age, gestational weight gain, and delivery mode. Notably, *HSD11B1* DMI remained independently associated with lower placental 11 β -HSD2 activity.

One of the most consistent patterns observed in the present study was the association between maternal weight-related variables and placental DMI markers. *HSD11B1*

DMI was positively associated with gestational weight gain, whereas *HSD11B2* island 1 and *HSD11B2* island 2 showed distinct and partly divergent correlation patterns with pre-pregnancy BMI and gestational weight gain. These associations were nominal and did not remain significant after FDR correction; therefore, they should be interpreted as exploratory. In addition, cord cortisol was positively correlated with gestational weight gain (%), whereas placental 11 β -HSD2 activity was negatively correlated with gestational weight gain. These observations suggest that maternal nutritional and metabolic conditions during pregnancy may be reflected in placental epigenetic variation at genes involved in glucocorticoid metabolism. The relationship between obesity and CpG-site methylation has been extensively described in non-pregnant populations [23–27]. This is also relevant in the context of foetal programming and the Barker hypothesis that an inadequate nutrient supply during foetal life may predispose individuals to the development of various diseases later in life [28]. Previous studies have linked maternal obesity and high-fat diet during pregnancy with increased risk of central nervous system abnormalities and psychiatric disorders in offspring, including depression, anxiety, and attention-deficit/hyperactivity disorder [29,30]. However, the present study did not include postnatal follow-up; therefore, our findings should not be interpreted as evidence of later developmental consequences. Rather, they suggest that maternal weight-related variables may be relevant to placental epigenetic variation in the glucocorticoid-pathway at term.

The separate analysis of two *HSD11B2* CpG islands indicated that methylation-related variation within this gene should not be treated as a single uniform signal. DMI values for *HSD11B2* island 1 and *HSD11B2* island 2 were not correlated with each other and showed distinct nominal associations with clinical variables. *HSD11B2* island 1 DMI was associated mainly with pre-pregnancy BMI and gestational weight gain (%), whereas *HSD11B2* island 2 DMI was associated with gestational weight gain (kg) and, in vaginal deliveries, with gestational age. Although these region-specific patterns did not remain significant after FDR correction, they may help generate hypotheses for future analyses of *HSD11B2* regulatory regions. These findings suggest that different regulatory regions within *HSD11B2* may be differentially related to maternal or perinatal factors. Because the analysed regions differ in their position

relative to the coding sequence and in their potential regulatory context, assessment of both regions may help avoid overlooking region-specific methylation patterns.

The clearest association observed in the adjusted models was the inverse relationship between *HSD11B1* DMI and placental 11 β -HSD2 activity. Because 11 β -HSD1 and 11 β -HSD2 have opposing roles in regulating local cortisol availability, this finding may reflect shared or coordinated regulation of placental glucocorticoid metabolism rather than an isolated effect of a single locus. Variation in *HSD11B1* DMI may therefore reflect broader placental regulatory state associated with the glucocorticoid pathway. However, this interpretation should be approached cautiously. The present study did not assess *HSD11B1* expression or enzymatic activity and therefore cannot determine whether *HSD11B1* DMI functionally modifies 11 β -HSD1 activity. Moreover, although 11 β -HSD1 and 11 β -HSD2 both participate in placental glucocorticoid metabolism, they have opposing catalytic roles and are not necessarily regulated in parallel [31,32]. Whole-thickness placental sampling may also capture differences in tissue composition or shared upstream regulatory influences rather than a direct mechanistic effect of *HSD11B1* methylation on 11 β -HSD2 activity.

Although several unadjusted associations were observed for cord cortisol, none of the analysed DMI markers remained independently associated with this outcome after adjustment. Although the unadjusted correlation between cord cortisol and *HSD11B2* island 1 DMI remained significant after FDR correction, this association was attenuated after covariate adjustment. By contrast, delivery mode remained independently associated with cord cortisol in all adjusted cord cortisol models, and gestational weight gain (%) remained significant in two of these models. Previous animal and human studies have reported associations of maternal stress, anxiety, depression, drug exposure, or tobacco smoke with decreased expression of placental *HSD11B1* and *HSD11B2* leading to increased foetal cortisol [33–39]. Increased *NR3C1* methylation has also been associated with infant salivary cortisol measures, suggesting a potential link between glucocorticoid receptor methylation and infant stress reactivity [40]. The present findings suggest, however, that cord cortisol at birth may be more strongly influenced by clinical and perinatal factors, including delivery-related physiology, gestational weight gain, and labour-related stress, than by DMI status at the selected

placental loci alone. Cord cortisol and placental 11 β -HSD2 activity should therefore be interpreted as related but biologically distinct outcomes. Cord cortisol reflects the foetal endocrine state at delivery and may be affected by acute perinatal conditions, whereas placental 11 β -HSD2 activity reflects the enzymatic capacity of the placenta to inactivate cortisol and therefore a more direct functional marker of placental glucocorticoid metabolism.

The absence of significant differences in DMI of *HSD11B1*, *HSD11B2*, or *NR3C1* according to delivery mode suggests that, within this cohort of term pregnancies, delivery mode was not associated with placental methylation status at the analysed loci. Thus, we did not observe evidence that acute perinatal stress associated with uterine contractions and vaginal delivery was reflected in broad delivery mode-related differences in placental DMI. Nevertheless, some correlations were observed within the vaginal delivery subgroup. *HSD11B1* DMI showed nominal associations with pre-pregnancy BMI, gestational weight gain (%), duration of uterine contractions, and epidural analgesia. These findings may suggest that labour-related or maternal factors are linked to variation in *HSD11B1* methylation in vaginal deliveries. However, they should be interpreted cautiously because of the smaller subgroup size and the non-random nature of labour-related variables, including epidural analgesia. Although delivery mode was not associated with placental DMI in our cohort, previous studies indicate that delivery mode is related to acute foetal or newborn endocrine stress responses, including differences in cord blood cortisol and catecholamine concentrations between vaginal, assisted vaginal, and caesarean deliveries [41,42]. The present data therefore support a cautious interpretation in which delivery mode may be more relevant to perinatal endocrine outcomes than to methylation variation at the analysed placental regions.

Several secondary findings were observed, although most were subgroup-specific and should be interpreted as exploratory. *HSD11B1* DMI was higher in placentas from female than male newborns. In contrast, we did not observe sex-related differences in *HSD11B2* DMI at either analysed CpG island, although our previous study reported sexual dimorphism in placental 11 β -HSD2 activity accompanied by corresponding differences in *HSD11B2* expression levels [43]. No consistent associations were

observed between DMI markers and newborn birth weight. This is relevant because previous placental methylation studies have linked methylation of glucocorticoid-pathway genes, including *HSD11B1* and *HSD11B2*, with foetal growth-related outcomes. For example, lower methylation at the *HSD11B1* CpG1 site was reported to be associated with increased risk of being classified as large for gestational age [20]. In the same study, *HSD11B1* expression was positively correlated with methylation at a single CpG site, whereas *HSD11B2* expression was negatively correlated with mean methylation across four promoter CpG sites. Together with previous evidence, these findings support the concept that 11 β -HSD-mediated regulation of foetal glucocorticoid exposure may contribute to foetal growth and development, with DNA methylation potentially participating in this regulatory process. Several studies have examined placental *HSD11B2* methylation in relation to foetal growth and pregnancy complications. Increased *HSD11B2* methylation has been reported in infants with intrauterine growth restriction [44], whereas decreased methylation has been observed in association with maternal pre-eclampsia [45]. In addition, reduced placental 11 β -HSD1 activity at birth has been linked to diminished postnatal growth in children born small for gestational age, persisting up to 7 years of age [46]. However, in the present cohort, associations between DMI markers and newborn anthropometric parameters were limited and not consistent across genes, loci, or delivery-mode subgroups. In the present study, we did not observe consistent associations between *HSD11B1* or *HSD11B2* DMI and newborn birth weight. However, when ponderal index was used as an anthropometric measure of body proportionality, *HSD11B2* island 1 DMI was positively correlated with newborn ponderal index in vaginal deliveries, whereas *NR3C1* DMI was negatively correlated with newborn ponderal index in the same group. Therefore, our findings do not provide strong evidence that methylation variation at the analysed regions is directly reflected in birth weight at term.

The strengths of this study include analysis of term placental tissue, assessment of several biologically relevant glucocorticoid pathway loci, separate evaluation of two *HSD11B2* CpG islands, and combined analysis of DMI markers with cord cortisol and placental 11 β -HSD2 activity. The use of multivariable models also helped distinguish unadjusted associations from findings that remained evident after adjustment. The

main limitation is the relatively small sample size, particularly for subgroup analyses, which limits the ability to detect modest differences related to delivery mode in placental DMI. In addition, most exploratory correlations did not remain significant after FDR correction, and therefore these findings should be interpreted as hypothesis-generating rather than confirmatory. This study was cross sectional and observational, therefore, causal inference is limited. Functional interpretation is also limited by the absence of *HSD11B1*, *HSD11B2*, and *NR3C1* mRNA or protein expression data, and by the lack of 11 β -HSD1 enzymatic activity measurements. In addition, postnatal cortisol assessment was not available. The cell-type composition of placental biopsies was not deconvolved; therefore, observed differences may partly reflect variation in trophoblast, stromal, or vascular cell proportions. We also did not stratify caesarean sections by indication, such as elective vs urgent caesarean section, and maternal smoking, gestational diabetes mellitus, hypertensive disorders, and maternal mental health were not incorporated as covariates. These factors may have confounded placental methylation status. Future studies in larger cohorts, including women undergoing urgent caesarean section and incorporating postnatal cortisol assessment, gene expression, enzyme activity and placental cell composition data, could help clarify the relationship between maternal or perinatal factors and glucocorticoid-pathway regulation in the placenta.

To conclude, in this term pregnancy cohort, delivery mode was not associated with DMI markers at the analysed regions of *HSD11B1*, *HSD11B2* (island 1 and island 2), or *NR3C1*. Maternal weight-related variables showed the most coherent nominal associations with *HSD11B1* and *HSD11B2* DMI, suggesting that maternal metabolic status may be relevant to placental epigenetic variation. Cord cortisol appeared to be more closely associated with clinical and perinatal factors, including gestational weight gain and delivery mode, than with DMI status at the investigated loci. In contrast, placental 11 β -HSD2 activity was independently associated with *HSD11B1* DMI. These findings are rather hypothesis-generating and require further validation in larger cohorts with integrated methylation, gene expression, enzyme activity, cell-composition data, maternal metabolic characteristics, and postnatal offspring outcomes.

4 Materials and Methods

4.1 Study design and participants

This study used the same cohort as previously described [20]. Placental samples were obtained at the 1st Department of Obstetrics and Gynecology, Centre of Postgraduate Medical Education (CMKP) in Warsaw, between July 2016 and January 2017. The study included 70 placentas from singleton term pregnancies, defined as gestational age 37–41 weeks, delivered vaginally or by caesarean section. The main exclusion criteria relevant to this analysis were multiple pregnancy, preterm birth, birth weight < 2500 g, low Apgar score, early neonatal intensive care admission, congenital or chromosomal abnormalities, major maternal diseases including diabetes or hypertension, smoking, alcohol use, placental malformation, and recent glucocorticosteroid or antibiotic treatment. Detailed inclusion and exclusion criteria have been reported previously [47]. Of the 70 placentas, 43 were obtained after caesarean section and 27 after vaginal delivery. Thirty caesarean sections were elective, whereas 13 were performed after the onset of regular uterine contractions. No caesarean section was performed because of foetal distress or prolonged labour.

4.2 Tissue collection

All placentas were delivered spontaneously within 45 min of foetal delivery. Cases requiring manual removal of the placenta or showing evidence of structural disruption were excluded. The decidual surface of each placenta was rinsed with sterile phosphate-buffered saline (PBS; pH 7.5). Using sterile, disposable surgical tools, three full-thickness tissue samples, approximately 1 cm³ each, were collected near the umbilical cord insertion site from the basal to the chorionic surface, while avoiding areas of calcification, infarction, or haemorrhage. Each sample was then thoroughly rinsed with PBS, snap-frozen in liquid nitrogen, and stored at −80°C until further analysis.

4.3 Genomic DNA isolation and bisulphite conversion

Genomic DNA was isolated from placental samples using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). RNase A (Blirt, Gdansk, Poland) was included during the isolation procedure. Bisulphite conversion of 1 µg input DNA was performed using the EpiJET Bisulfite Conversion Kit (Thermo Scientific, Waltham, MA, USA). The concentration of bisulphite-converted DNA was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), assuming that an absorbance of 1.0 at 260 nm corresponds to 40 µg/mL DNA. DNA concentration was adjusted to 10 ng/µL with PCR-grade water. Samples were stored at 4°C until analysis.

4.4 Methylation-specific real-time qPCR

MethPrimer [48] was used to identify CpG islands or CpG-containing regions and to design primers for methylation-specific quantitative PCR (MS-qPCR) (Figure 7). Primer sequences specific for methylated (M) and unmethylated (U) templates, together with amplicon details, are provided in Supplementary Table S1. MS-qPCR was performed in duplicate using SG qPCR Master Mix (2X) (EurX, Gdańsk, Poland), 0.5 µM primers (Thermo Fisher Scientific, Glasgow, UK), and 10–15 ng of bisulphite-converted DNA in a total reaction volume of 10 µL. Reactions were run on a LightCycler 480 Instrument II (Roche Diagnostics Ltd., Rotkreuz, Switzerland). Amplification was performed for 45 cycles with target-specific annealing temperatures (51–60°C), followed by melting curve analysis from 65°C to 95°C. Full cycling conditions are provided in Supplementary Table S2. Amplification conditions were optimized to achieve primer efficiencies of 80–100%. Primer sets specific for methylated and unmethylated templates were required to have similar efficiencies, with a difference of < 10%. No-template controls and non-bisulphite-modified genomic DNA were included to assess reagent purity and primer specificity, respectively. To estimate the relative abundance of unmethylated DNA, mean crossing threshold values obtained for methylated (C_{t_M}) and unmethylated (C_{t_U}) reactions were used to calculate the demethylation index (DMI), as described by Akirav et al. [49]: $DMI = 2^{(C_{t_M} - C_{t_U})}$. DMI values were used for downstream statistical analyses. A DMI < 1 indicates predominantly methylated

status, whereas a $\text{DMI} > 1$ indicates predominantly demethylated or unmethylated status of the analysed sequence. Thus, higher DMI reflects a greater relative abundance of unmethylated template and should not be interpreted as higher methylation.

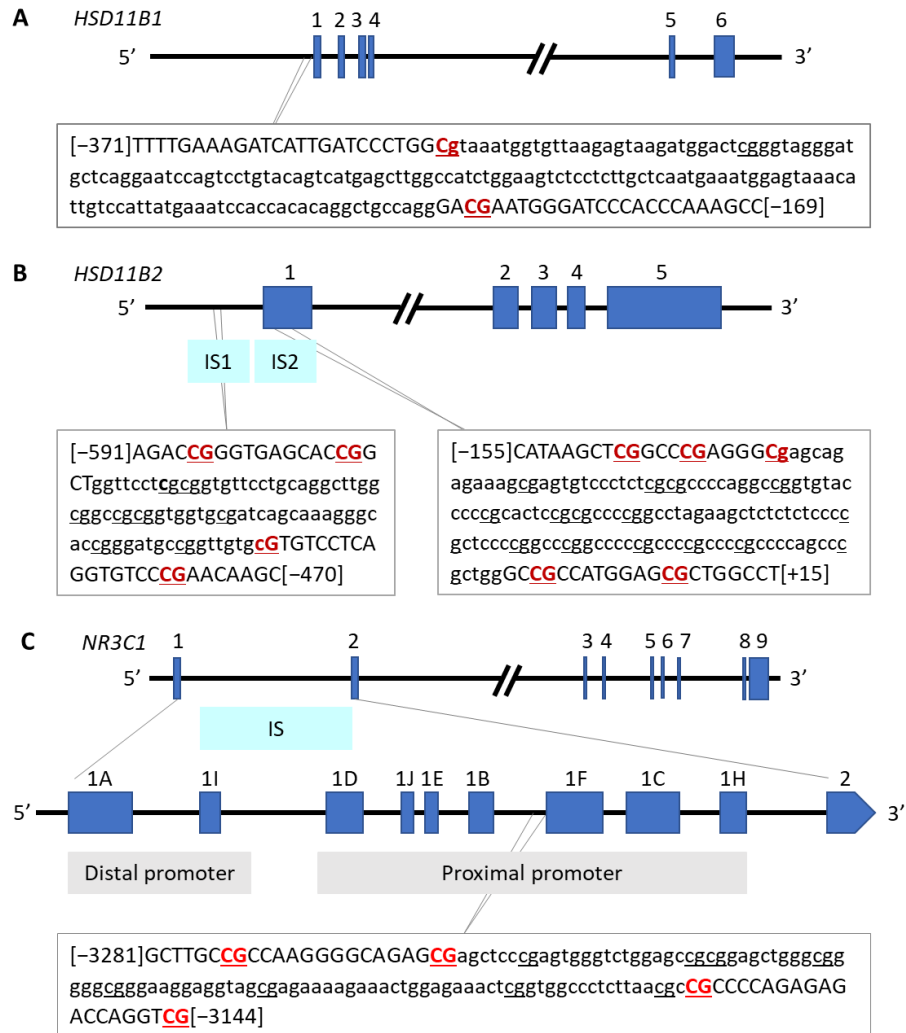


Fig. 7. Analysed CpG regions in *HSD11B1*, *HSD11B2*, and *NR3C1*. Gene structure and location of CpG sites analysed by methylation-specific quantitative PCR (MS-qPCR). The nucleotide sequences of the MS-qPCR amplicons are shown for *HSD11B1* (A), *HSD11B2* (B), and *NR3C1* (C). The position of each amplified region relative to the translation start site is indicated in brackets, and primer-binding sites are shown in uppercase. CpG dinucleotides within the amplicon are underlined, whereas CpG dinucleotides within the primer sequences are marked in red. Exons are shown as dark blue boxes, and CpG islands (IS) are shown as light blue boxes.

4.5 Determination of 11 β -HSD2 activity and cord blood cortisol

Placental 11 β -HSD2 activity and cord blood cortisol concentrations were determined as previously described [43]. The specific activity of 11 β -HSD2 was expressed as pg cortisone/mg protein/min, corresponding to the amount of cortisone formed per minute per milligram of protein. Cord blood cortisol concentration was expressed as μ g/dL.

4.6 Statistical analysis

Data were analysed using STATISTICA 13 software (StatSoft Inc., Cracow, Poland). Numerical variables, including DMI values for *HSD11B1*, *HSD11B2* island 1 and island 2, *NR3C1*, and numerical clinical characteristics were summarized as medians with interquartile ranges (IQR; 25th–75th percentiles). Categorical variables were summarized as counts and percentages. The Mann-Whitney U test was used to compare DMI values between delivery modes, between placentas from male and female newborns, as well as between epidural analgesia and no analgesia in vaginal deliveries. Spearman correlation coefficients (r) were used to assess associations between DMI values and numerical clinical characteristics, as well as correlations among *HSD11B1*, *HSD11B2* island 1 and island 2, *NR3C1* DMI values. Separate multivariable linear regression models were constructed for cord cortisol and placental 11 β -HSD2 activity. Each model included one DMI variable at a time together with the prespecified covariates: gestational age, gestational weight gain (%), and delivery mode (vaginal delivery vs caesarean section). Predictors were entered simultaneously using the forced-entry method. Model diagnostics included visual assessment of residual distribution and residuals-versus-fitted plots. For all analyses, $P < 0.05$ was considered statistically significant. Nominal P -values were additionally adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) procedure. FDR correction was applied separately within predefined analysis groups: total group, caesarean section subgroup, and vaginal delivery subgroup; for the study group characteristics, correction was applied across group-comparison tests.

Supplementary information: Supplementary materials (DOCX file) with Tables S1–S4.

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Data availability: Data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate: The study was conducted in accordance with the Declaration of Helsinki at the 1st Department of Obstetrics and Gynaecology, Centre of Postgraduate Medical Education (CMKP), Warsaw, Poland, and was approved by the Bioethics Committee of CMKP (Approval No. 8/PB/2014). All participants provided written informed consent.

Competing interests: The authors declare no competing interests.

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