Original Article

DNA methylation of the glucocorticoid receptor gene promoter in the placenta is associated with blood pressure regulation in human pregnancy

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Background: Blood pressure (BP) regulation during pregnancy is influenced by hormones of placental origin. It was shown that the glucocorticoid system is altered in hypertensive pregnancy disorders such as preeclampsia. Epigenetic mechanism might influence the activity of genes involved in placental hormone/hormone receptor synthesis/action during pregnancy.

Method: In the current study, we analyzed the association of 5′-C-phosphate-G-3′ (CpG) site methylation of different glucocorticoid receptor gene (*NR3C1*) promoter regions with BP during pregnancy. The study was performed as a nested case-control study ($n = 80$) out of 1045 mother/child pairs from the Berlin Birth Cohort. Placental DNA was extracted and bisulfite converted. Nested PCR products from six *NR3C1* proximal promoter regions [glucocorticoid receptor gene promotor region B (GR-1B), C (GR-1C), D (GR-1D), E (GR-1E), F (GR-1F), and H (GR-1H)] were analyzed by next generation sequencing.

Results: NR3C1 promoter regions GR-1D and GR-1E had a much higher degree of DNA methylation as compared to GR-1B, GR-1F or GR-1H when analyzing the entire study population. Comparison of placental *NR3C1* CpG site methylation among hypotensive, normotensive and hypertensive mothers revealed several differently methylated CpG sites in the GR-1F promoter region only. Both hypertension and hypotension were associated with increased DNA methylation of GR-1F CpG sites. These associations were independent of confounding factors, such as family history of hypertension, smoking status before pregnancy and prepregnancy BMI. Assessment of placental glucocorticoid receptor expression by western blot showed that observed DNA methylation differences were not associated with altered levels of placental glucocorticoid receptor expression. However, correlation matrices of all *NR3C1* proximal promoter regions demonstrated different correlation patterns of intraregional and interregional DNA methylation in the three BP groups, putatively indicating altered transcriptional control of glucocorticoid receptor isoforms.

Conclusion: Our study provides evidence of an independent association between placental *NR3C1* proximal promoter methylation and maternal BP. Furthermore, we observed different patterns of *NR3C1* promoter methylation in normotensive, hypertensive and hypotensive pregnancy.

Keywords: DNA methylation, epigenetics, glucocorticoid receptor, hypertension, hypotension, *NR3C1* gene, placenta, pregnancy

Abbreviations: GR, glucocorticoid receptor; GR-1B, glucocorticoid receptor gene promotor region B; GR-1C, glucocorticoid receptor gene promotor region C; GR-1D, glucocorticoid receptor gene promotor region D; GR-1E, glucocorticoid receptor gene promotor region E; GR-1F, glucocorticoid receptor gene promotor region F; GR-1H, glucocorticoid receptor gene promotor region H; MAP, mean arterial blood pressure; *NR3C1*, glucocorticoid receptor gene

INTRODUCTION

Hypertensive disorders of pregnancy (HDP) are a common pregnancy complication. HDP encompass several diseases, including chronic hypertension, gestational hypertension, preeclampsia and eclampsia [1]. According to the WHO, the prevalence of HDP is as high as 14% [2]. HDP is associated with adverse pregnancy outcomes for both, mother and fetus [3]. On the other hand, hypotension during pregnancy is also associated with impaired pregnancy outcomes [4].

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Blood pressure (BP) regulation during pregnancy is mediated at least partially by hormonal factors [5]. A crucial hormone in this regard is cortisol [6]. The production of cortisol increases several times during pregnancy [6]. It was reported that cortisol catabolism is more pronounced in HDP compared with normal pregnancies [7]. In particular cortisol metabolism in the human placenta is linked to HDP in humans [8]. Our knowledge of a putative association between cortisol metabolism and hypotension during pregnancy, however, is limited so far. Cortisol likewise plays a key role in organ development of the growing fetus. This seems to be in particular important for the development of the human brain [9].

Cortisol effects are mediated by the glucocorticoid receptor. After binding cortisol, the cortisol–glucocorticoid receptor complex has the ability to bind glucocorticoid response elements in the promoter region of cortisol responsive genes. Together with transcription factors, this cascade leads to an increased or decreased gene expression of – for example – genes involved in BP regulation [10]. Henceforth, in addition to circulating cortisol levels, the degree of glucocorticoid receptor expression is an important factor in determining the effect of cortisol on BP [11]. The glucocorticoid receptor is expressed in the placenta [12]. The interaction between placental glucocorticoid receptor and cortisol affects placental growth and development [13], and supports the central placental function as a transfer organ between mother and fetus [14]. Impaired glucocorticoid receptor expression in the placenta can affect fetal growth and development [13]. Current literature furthermore suggests an involvement of glucocorticoid receptor expression in preeclampsia [15]. Modulation of gene expression in the placenta may be mediated by epigenetic mechanisms, including DNA methylation [16,17]. It was reported that BP is correlated with DNA methylation patterns in placental genes [18]. Specifically for the glucocorticoid receptor gene (*NR3C1*), it had been reported that stress during pregnancy can influence the methylation level of this gene [19]. However, there is no study yet that investigated if there is a correlation between maternal BP as another parameter determining the prenatal environment and the methylation level of *NR3C1*.

NR3C1 contains two distinguished groups of promoters termed distal and proximal promoters [20]. The group of proximal promoters consists of several different promoter regions, including glucocorticoid receptor gene promotor region D (GR-1D), glucocorticoid receptor gene promotor region E (GR-1E), glucocorticoid receptor gene promotor region B (GR-1B), glucocorticoid receptor gene promotor region F (GR-1F), glucocorticoid receptor gene promotor region C (GR-1C) and glucocorticoid receptor gene promotor region H (GR-1H) [21]. Proximal promoter regions are potential targets for epigenetic modifications, as they are located in a 5′-C-phosphate-G-3′ (CpG) island [21]. There is growing evidence that CpG island promoter methylation is important for the modulation of gene expression [22]. Moreover, the methylation level of proximal promoters is sensitive to factors determining the prenatal environment, including maternal BP [21] The purpose of this study was to explore the correlation between maternal BP and the methylation level of placental *NR3C1* proximal promoter regions and to identify potential patterns of glucocorticoid receptor promoter methylation that are specific for hypertensive or hypotensive pregnancies.

MATERIALS AND METHODS

Clinical study

This nested case–control study is a part of Berlin Birth Cohort study [23]. The study was approved by the Institutional Review Board of the university hospital of Charité, Berlin, Germany [24,25]. The study was conducted in 1045 placental samples from mothers [26] who delivered in the obstetrics department of Campus Charité Mitte, Berlin, Germany. Structured interviews were carried out after obtaining written consent to take part in the study. A pregnancy health document (Mutterpass) was used to extract relevant data such as diabetes history, hypertension history and smoking status. Gestational age was calculated based on the last menstruation period. Routine antenatal examinations were used to collect SBP and DBP data. For all individuals, mean SBP and DBP were calculated from all available readings. Eighty placenta samples were chosen as a representative of 1045 placenta samples, considering SBP and DBP of the mother in the 3rd trimester of pregnancy. Biometric parameters such as sex of the newborn, birth size and APGAR score were documented during the postnatal examination. A standardized placenta sample (one cotyledon from similar locations) was obtained, immediately frozen and stored at −20 °C. A sample of chorionic villi was acquired from the cotyledon samples for DNA and protein extraction.

DNA isolation and bisulfit treatment

DNA extraction was performed using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the recommended procedure by the manufacturer. RNA interference was minimalized by adding RNAse during the DNA isolation process. DNA solution was then analyzed with a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific, Renfrew, UK) to measure quality and quantity of the DNA. DNA and protein content were measured as absorbance at 260 and 280 nm, respectively. Only samples with 260/280 nm absorbance ratio between 1.9 and 2.1 were considered to be processed further. Bisulfite treatment was performed using EZ-96 DNA methylation kit following the protocol from the manufacturer for an amount of maximal 500-ng DNA in each sample solution.

PCR and illumina sequencing

Nested PCR was performed to prepare the amplicons for sequencing on an Illumina MiSeq. PCR primers were designed based on the selected areas of *NR3C1* which included the proximal promoters GR-1D, GR-1E, GR-1B, GR-1F, GR-1C and GR-1H (Fig. 1; Supplemental Table 1, http://links.lww.com/HJH/A800). The first PCR was conducted using an outer primer (Supplemental Table 1, http://links.lww.com/HJH/A800) by applying the following protocol: predenaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, annealing at approximate melting temperature of the outer promoter for 30 s (Supplemental

FIGURE 1 Schematic diagram of selected glucocorticoid receptor gene CpG sites which were explored in this study. Five PCR products from six different glucocorticoid receptor gene proximal promoter areas were designed in this study. The corresponding sequences for designed primers are indicated in bold characters. Numbers in the box for every PCR product indicate the relative position of the last base in the PCR product to the transcription-starting site. Glucocorticoid receptor gene promotor region D, glucocorticoid receptor gene promotor region E, glucocorticoid receptor gene promotor region B/1F, glucocorticoid receptor gene promotor region F/glucocorticoid receptor gene promotor region C and glucocorticoid receptor gene promotor region H are primers for amplification of glucocorticoid receptor gene promotor region D, glucocorticoid receptor gene promotor region E, glucocorticoid receptor gene promotor region B/1F, glucocorticoid receptor gene promotor region F/1C and glucocorticoid receptor gene promotor region H of the glucocorticoid receptor gene proximal promoter, respectively. Forward (Fwd) or reverse (Rev) primers are indicated. The first translation site is located in the exon-2 (arrow above the box). The numbering of CpG sites was assigned based on a previous study.

Table 1, http://links.lww.com/HJH/A800) and 72 °C for 4 min for extension step. Final extension was performed at 72 °C for 10 min. The obtained amplicons were further used as templates for a second PCR using inner primer (Supplemental Table 1, http://links.lww.com/HJH/A800). The inner primers are completed with 10 individual barcodes for the sequencing purpose. Ten barcodes which were used were TCGCAGG, CTCTGCA, CCTAGGT, GGATCAA, GCAAGAT, ATGGAGA, CTCGATG, GCTCGAA, ACCAACT and CCGGTAC. Five different nested PCR products from six *NR3C1* proximal promoter areas were designed in this study (Fig. 1). After adjusting the concentration of the nested PCR products to equal amounts using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, California, USA), amplicon pools were prepared for sequencing with AMPure XP beads (Beckman Coulter Genomics, Danvers, Massachusetts, USA). Size and quantity of the amplicon library were assessed on a Fragment Analyzer (AATI; Analytical Technologies, Inc., Ankeny, Iowa, USA). The libraries were clustered to a density of approximately 800 K/mm^2 and sequenced with 2× 300v3 paired-end MiSeq runs. Image analysis, base calling and data quality assessment were performed on the MiSeq instrument.

Data processing

The sequence reads were merged, trimmed and demultiplexed using CLC Genomics Workbench 7 software (Qiagen) using the default parameters with 200 bp as minimum length. Subsequently, at least 20 processed reads were analyzed for their DNA methylation level quantification using the BiQ Analyzer HT software (Max-Planck-Institut for Informatics, Computational Biology and Applied Algorithmics, Germany) [27].

Western blots

Western blots were performed as previously described [28]. Chorionic villi samples were pulverized in a metal mortar after cooling in liquid nitrogen. Protein extraction was performed using a urea/thiourea buffer [2 mol/l thiourea, 7 mol/l urea, 2% SDS, 1% dithiothreitol and protease inhibitor (Complete Mini, Cat. No.: 11 697 498 001; Roche)]. Protein extracts were separated by SDS–PAGE employing a 10% polyacrylamide gel. After electrophoresis, gels were blotted onto nitrocellulose membranes (AmershamTM HybondTM ECL; GE Healthcare, Little Chalfont, UK) using a Biorad Trans-Blot semidry blotter and transfer buffer (184 mmol/l glycine, 24 mmol/l Tris, 20% methanol). The primary antibody incubation (anti-glucocorticoid receptor, sc-8992; Santa Cruz Biotechnology, Dallas, Texas, USA) was performed at 4 °C overnight. After washing with TBS-T, the membrane was incubated in secondary antibody (horseradish-peroxidase conjugated antigoat IgG; sc-2020; Santa Cruz Biotechnology) for 1 h at room temperature. Protein signals were developed using enhanced chemiluminescence solution. As a housekeeping protein expression of glyceraldehyde 3-phospate dehydrogenase (GAPDH) was used (anti-GAPDH, sc-32233; Santa Cruz Biotechnology). The GAPDH signal was developed using an alkaline phosphatase-conjugated secondary antibody (antimouse IgG,

TABLE 1. Characteristics of the explored mother–child pairs data

Parameter	Hypotension (27)	Normotension (33)	Hypertension (20)	*P*
Gestational age at delivery (week)	39.0 ± 1.8	38.9 ± 1.4	38.3 ± 2.0	0.290
Age of the mother (year)	30.7 ± 7.6	29.1 ± 5.2	30.6 ± 4.6	0.524
Prepregnancy BMI (kg/m^2)	21.0 ± 3.0	23.6 ± 4.3	28.7 ± 6.5	<0.0001
SBP 3rd trimester (mmHg)	96.8 ± 9.2	118.6 ± 13.8	144.9 ± 7.7	<0.0001
DBP 3rd trimester (mmHg)	55.0 ± 3.8	72.6 ± 9.3	89.9 ± 4.2	<0.0001
Smoking before pregnancy (%)	25.9	45.5	45.0	0.244
Hypertension in family (%)	33.3	39.4	80.0	0.003
Diabetes in family (%)	26.1	26.7	61.5	0.055
Ethnicity, white/nonwhite	88.9	93.9	100.0	0.298
Child birth weight (g)	3342.6 ± 636.5	3249.1 ± 392.5	3098.8 ± 649.9	0.331
Child head circumference (cm)	34.6 ± 1.7	34.6 ± 1.3	33.8 ± 1.7	0.212
Child birth length (cm)	50.8 ± 3.4	50.0 ± 3.4	49.4 ± 2.8	0.313
Ponderalindex	25.3 ± 2.2	26.9 ± 9.9	25.4 ± 2.0	0.586
APGAR score 5 min	9.3 ± 1.0	9.3 ± 1.3	8.6 ± 2.1	0.296
APGAR score 10 min	9.8 ± 0.5	9.6 ± 0.7	9.2 ± 1.4	0.114
Sex of the child, male/female	44.4/55.6	45.5/54.5	40.0/60.0	0.924

Data are given as mean ± SD or %.

sc-2008; Santa Cruz Biotechnology) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride (BCIP/NBT) as substrate. The developed membranes were digitalized using a 600 dpi scanner resolution and analyzed with AlphaEaseFCTM (Alpha Innotech, San Leandro, California, USA).

Statistical analysis

Statistical analyses were performed using SPSS version 20 (SPSS Inc., Chicago, IL, USA). BP grouping was performed based on previously published criteria [29,30]. Gestational hypertension was defined as mothers who had mean SBP at least 140 or DBP at least 90 mmHg in the second half of pregnancy, but no history of hypertension prior to pregnancy. The control group accommodated mothers who had SBP between 90 and 140 and DBP between 60 and 90 mmHg. Hypotension was defined as SBP less than 90 or DBP less than 60 mmHg in the second half of pregnancy, but no history of hypotension prior to pregnancy (for details regarding distribution and cutoffs of SBP and DBP see Supplemental Fig. 1, http://links.lww.com/HJH/A800). Unpaired *t* test or Mann–Whitney *U* test was carried out to compare continuous parameters, if two groups were compared. Normal distribution of the data was tested using the Kolmogorov–Smirnov test. Based on normal distribution of the data analysis of variance (ANOVA) analysis followed by a Tukey honestly significant difference (HSD) post-hoc test or Kruskal–Wallis followed by Dunn–Bonferroni post-hoc test was used to compare continuous parameters between the three BP groups. Comparisons of categorical parameters among different BP groups were performed using chi-squared test. Independent association analysis between CpG site methylation and BP groups was performed by using multinominal logistic regression models adjusted for established factors influencing BP, that were found to be significantly correlated to DBP or SBP in the analyzed cohort (Supplemental Table 3, http://links.lww.com/HJH/A800). Bar graph calculation and compilation was performed using Graphpad Prism 5 (GraphPad Software Inc., La Jolla, California, USA).

RESULTS

Comparison analyses demonstrated no statistically significant differences in regards to clinically important parameters between the sample population ($n = 1045$) and the 80 selected samples of the representative cohort (Supplemental Table 2, http://links.lww.com/HJH/A800). These results indicate that the selected 80 samples can be used as a representation of the whole sample population. Table 1 displays detailed descriptive statistics of the 80 selected mothers and their newborns. The three BP groups consisted of 27 hypotensive, 33 normotensive and 20 hypertensive mothers (Table 1). ANOVA analysis followed by a Tukey HSD post-hoc test statistically substantiated BP differences in the 3rd trimester of pregnancy between the three groups [SBP ($P < 0.0001$) and DBP ($P < 0.0001$)]. Prepregnancy BMI was also significantly ($P < 0.0001$) different among the BP groups. No parameter of the newborns was found to be different among the three BP groups.

Figure 2 displays the degree of DNA methylation among all CpG sites analyzed in this study. Generally, GR-1D and GR-1E showed more DNA methylation compared with GR-1B, GR-1F or GR-1H. DNA methylation of GR-1C could not be analyzed in this study because of a too small number of reads in the next generation sequencing analysis. Comparison of the CpG site methylation level revealed several differently methylated CpG sites of GR-1F among the three BP groups. However, no significant differences were observed at any of the other studied glucocorticoid receptor CpG sites. Comparison of GR-1F CpG site methylation levels among the three BP groups showed a significantly higher DNA methylation of GR-1F CpG sites 1, 2, 12, 16, 20, 24 and 26 in placentas from hypertensive mothers compared with normotensive mothers (Fig. 3). Moreover, the levels of DNA methylation of GR-1F CpG sites 1, 3, 12, 12 and 20 were significantly higher in hypotensive compared with normotensive mothers.

To demonstrate that DNA methylation of GR-1F is independently associated with BP, multinominal logistic regression analyses were performed and adjusted for well known confounders impacting on BP that were

TABLE 2. Multinominal logistic regression analysis of the association between CpG site methylation in glucocorticoid receptor gene promotor region F and blood pressure group (dependent variable)

Methylation at CpG position	Blood pressure group	B	SE	*P*	Exp (B)	95% CI for exp (B)	
						Min	Max
1	Hypotension[a]	0.33	0.20	0.092	1.39	0.95	2.05
	Hypertension[a]	0.44	0.20	**0.029**	1.55	1.05	2.29
2	Hypotension[a]	0.22	0.15	0.140	1.25	0.93	1.69
	Hypertension[a]	0.42	0.16	**0.010**	1.52	1.11	2.10
3	Hypotension[a]	0.24	0.09	**0.006**	1.27	1.07	1.51
	Hypertension[a]	0.07	0.10	0.488	1.07	0.89	1.29
12	Hypotension[a]	0.37	0.18	**0.040**	1.44	1.02	2.05
	Hypertension[a]	0.62	0.20	**0.002**	1.86	1.26	2.73
16	Hypotension[a]	0.36	0.22	0.095	1.43	0.94	2.19
	Hypertension[a]	0.74	0.25	**0.003**	2.10	1.29	3.42
20	Hypotension[a]	0.18	0.09	**0.046**	1.19	1.00	1.42
	Hypertension[a]	0.26	0.09	**0.005**	1.30	1.08	1.55
24	Hypotension[a]	0.12	0.15	0.453	1.12	0.83	1.52
	Hypertension[a]	0.46	0.18	**0.012**	1.58	1.11	2.25
26	Hypotension[a]	0.07	0.13	0.570	1.08	0.83	1.39
	Hypertension[a]	0.32	0.15	**0.026**	1.38	1.04	1.83

CI, confidence interval.
[a]Normotension was set as reference for these parameters. The models were confounded for: history of hypertension, age of the mother and prepregnancy BMI. Bold values indicate $P < 0.05$.

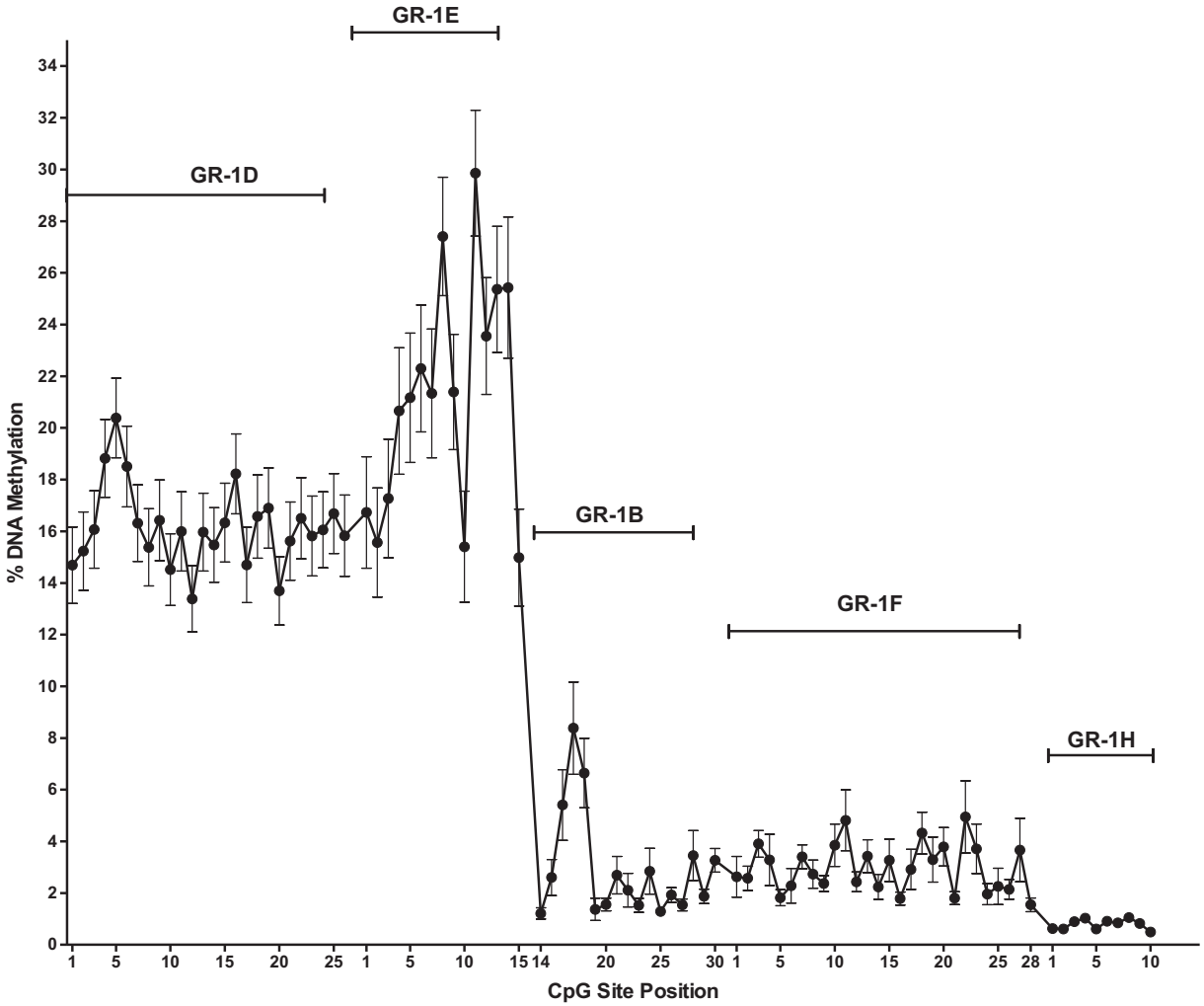

FIGURE 2 Methylation level of several CpG sites in five glucocorticoid receptor gene proximal promoters area. Different proximal promoters of glucocorticoid receptor gene showed different average methylation levels. Error bars represent SEMs. Graphs represent mean ± SEM.

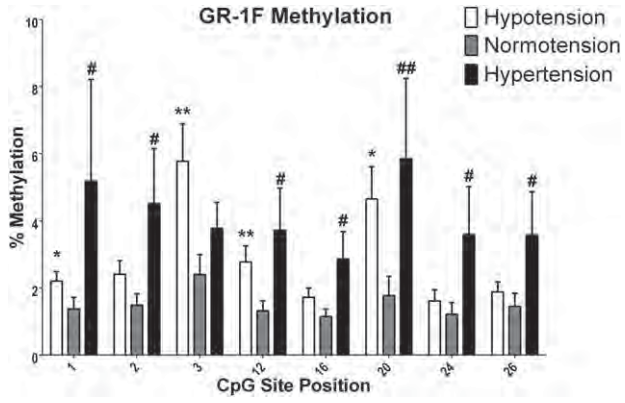

FIGURE 3 Mean methylation comparison of several CpG sites on glucocorticoid receptor gene promotor region F among blood pressure groups in univariate analysis. Only CpG sites with significantly different mean methylation level were shown. # and ## indicate comparison between normotension and hypertension with $P < 0.05$ and $P < 0.01$, respectively. * and ** indicate comparison between normotension and hypotension with $P < 0.05$ and $P < 0.01$, respectively. Error bars represent SEMs.

significantly correlated to DBP or SBP in the analyzed cohort (history of hypertension, smoking before pregnancyand prepregnancy BMI; Supplemental Table 3, http://links.lww.com/HJH/A800). Family history of diabetes was also correlated to DBP and SBP. As 14 values were missing in this variable and results were not altered by its inclusion to the model (data not shown), we did not include the variable to the model. Comparing CpG site methylation between the hypertensive and the normotensive group demonstrated an independent positive association of GR-1F CpG sites 1 ($P < 0.05$), 2 ($P < 0.05$), 12 ($P < 0.01$), 16 ($P < 0.01$), 20 ($P < 0.01$), 24 ($P < 0.05$) and 26 ($P < 0.05$) with hypertension. Compared with the normotensive group, the hypotensive group was independently associated with increased methylation of GR-1F CpG sites, 3 ($P < 0.01$), 12 ($P < 0.01$) and 20 ($P < 0.05$) (Table 2).

To assess if placental glucocorticoid receptor expression is affected by the observed increases in GR-1F CpG site methylation in hypotensive and hypertensive mothers, Western blots were performed. There were no significant differences in the level of placental glucocorticoid receptor expression among the three BP groups (Fig. 4). Furthermore, there was no significant correlation between GR-1F CpG sites and placental glucocorticoid receptor expression (data not shown). To investigate whether the degree of methylation of a given individual CpG site is correlated with other CpG sites within the same and also within different proximal promoter regions, spearman correlation matrices were calculated for each BP group. Resulting Spearman's ρs were plotted as heat maps separately for each BP group (Figs. 5–7) and average correlation coefficients for all possible regional interactions were calculated (see inserted diagram in each figure). Strong intraregional correlations were found in all three BP groups for GR-1D methylation (average spearman's $\rho = 0.9$) in all three groups. GR-1E methylation also displayed a strong intraregional correlation in the normotensive group (spearman's $\rho = 0.8$) which was less accentuated in the hypotensive (spearman's $\rho = 0.6$) and the hypertensive (spearman's $\rho = 0.5$) group. A more moderate intraregional correlation was observed for GR-1F methylation (spearman's $\rho = 0.5–0.6$ in all groups). In regards to interregional correlation of *NR3C1* proximal promoters, different patterns were observed in the three BP groups. Although hypotensive mothers displayed moderate (average spearman's ρ of the whole regions) to strong (correlations of clusters within promoter regions) negative correlations between GR-1B and GR-1D (average spearman's $\rho = -0.3$), and between GR-1D and GR-1F (average spearman's $\rho = -0.4$), these correlation patterns were not observed in normotensive or hypertensive mothers.

DISCUSSION

We provide evidence of an independent association between placental CpG island methylation pattern of the *NR3C1* promoter region GR-1F and BP regulation during pregnancy. Our findings are in agreement with data coming from nonpregnant hypertension studies showing that BP regulation can be influenced by *NR3C1* expression [31]. Moreover, our study provides evidence for different patterns of placental *NR3C1* promoter methylation in normotensive, hypertensive and hypotensive pregnancy.

The current study is the first to evaluate a potential association of maternal BP during pregnancy and methylation of *NR3C1* proximal promoter CpG sites. It is important to note that the findings of our study are independent of known confounding factors/risk factors of hypertensive disorders during pregnancy, such as family history of hypertension, smoking status and BMI (Table 2).

The result of this study reinforces the impact of the GR-1F methylation region in association with environmental factors as it has been reviewed previously [21].

However, we did not see a correlation between GR-1D promoter region methylation and BP regulation during pregnancy as it was seen by Hogg *et al.* [15]. There are several reasons that might explain this. Hogg *et al.* [15] focused on the comparison of healthy pregnant women and preeclamptic women. We on the other hand compared pregnant women with normal BP with pregnant women developing high BP during pregnancy independently from the degree of urinary protein excretion. The ethnic background of the study population analyzed by Hogg *et al.*, [15] analyzed at the University of British Columbia, Vancouver, British Columbia, Canada did display differences to the current study in regards to a larger proportion of Asian women (30%). Moreover, feeding habits of pregnant women in Berlin and Vancouver might also be different. It is for example very likely that the vitamin D status differed in both cohorts. Vitamin D during pregnancy, however, has a huge impact on pregnancy outcome [32]. Moreover, there are methodical differences that might also contribute at least partially to the differences seen in both studies. We analyzed six *NR3C1* proximal promoter regions (GR-1D, GR-1E, GR-1B/GR-1F, GR-1F/GR-1C and GR-1H) by next generation sequencing, whereas Hogg *et al.* [15] studied DNA methylation by bisulfite pyrosequencing.

A novelty of the current study is the inclusion of mothers experiencing hypotension during pregnancy. Interestingly the association between maternal BP and GR-1F methylation displayed a u-shaped behavior, with increased GR-1F methylation found in both mothers with hypotension and

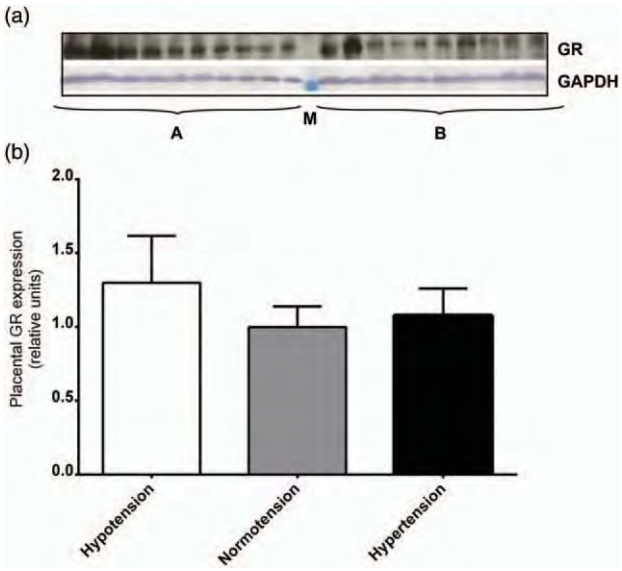

FIGURE 4 (a) Representative picture of glucocorticoid receptor western blot result and its corresponding housekeeping gene. On the left side (A), five different amounts of placental protein standard solution were pipette in two replicates, then a molecular weight marker (M), followed by individual protein samples as well in two replicates (B). Molecular weights were measured at 94 kDa for glucocorticoid receptor and at 37 kDa for glyceraldehyde 3-phospate dehydrogenase. (b) Placental expression of the glucocorticoid receptor among hypotensive, normotensive and hypertensive mothers. There were no significant differences in placental glucocorticoid receptor expression among the three blood pressure groups. Data shown ± SEM. GAPDH, glyceraldehyde 3-phospate dehydrogenase; GR, glucocorticoid receptor.

hypertension. Given a general lack of literature, it is hard to interpret this – at first sight – counterintuitive result. However, especially for glucocorticoid signaling and glucocorticoid receptor function, u-shaped and inverted u-shaped relationships were shown [33]. For example, memory retrieval is impaired, both, at very low as well as very high cortisol levels, but not at intermediate levels [34]. Such u-shaped effects of cortisol might be caused by its function through several receptor subtypes, which bear specific characteristics such as accessibility, affinity, desensitization or triggered signaling cascade [33]. Furthermore, interaction of cortisol with other receptors, such as the mineralcorticoid receptor, and its metabolism by 11-b-hydroxysteroid dehydrogenases might further contribute to u-shaped effects of cortisol [33]. Given the u-shaped behavior of glucocorticoid receptor signaling, it might be possible that methylation processes in *NR3C1* follow comparable patterns. Cell culture experiments in endothelial cells have demonstrated that glucocorticoid receptor signaling impacts on DNA methylation of *NR3C1* promoter sites [35]. Moreover, it was shown that depending on glucocorticoid sensitivity, different patterns of *NR3C1* promoter methylation emerge [35].

Until now, there have been no comparable studies that investigated associations between, both, hypotension and hypertension and *NR3C1* 1F promoter methylation. However, there are several studies that demonstrated associations between lower BP levels and higher methylation. Tempel *et al*. [36] showed in a cohort of healthy individuals that decreased baseline BP was associated with increased overall *NR3C1* 1F promoter methylation levels in DNA extracted from whole blood. Mata-Greenwood *et al*., who investigated the impact of glucocorticoid receptor signaling on *NR3C1* 1F promoter methylation levels in dexamethasone resistant and dexamethasone sensitive human umbilical vein endothelial cells, demonstrated increased 1F promoter methylation in dexamethasone resistant cells. Significantly, mothers from whom these cells were harvested displayed significantly lower SBP levels and

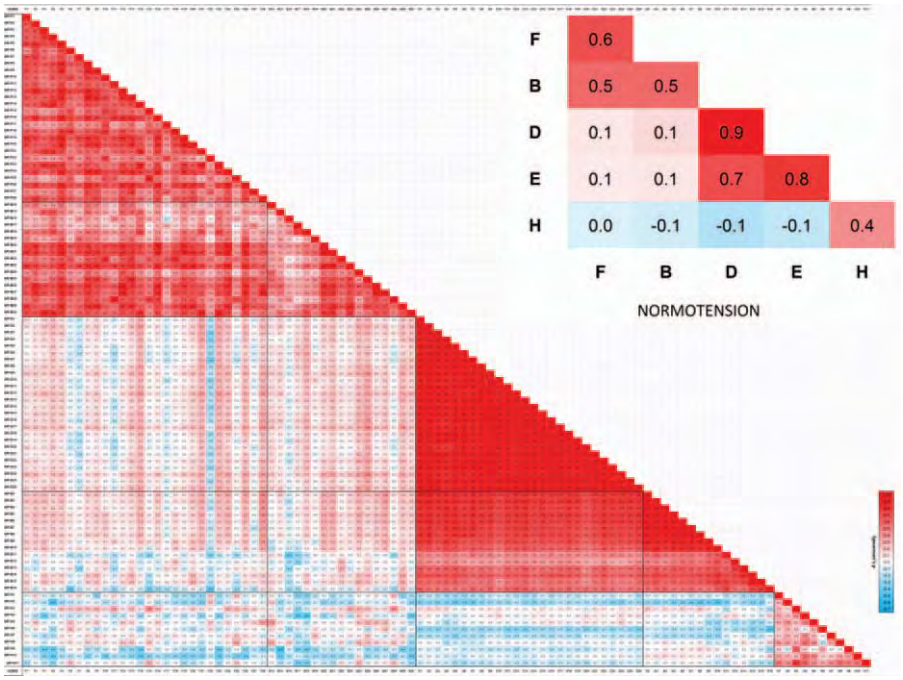

FIGURE 5 Correlation matrix of glucocorticoid receptor gene promoter region DNA methylation (Spearman's ρ shown as heat map). Correlation coefficients of intraregional and interregional glucocorticoid receptor gene proximal promoter CpG site methylation *in normotensive mothers* (red indicates positive, blue indicates negative correlations). The inserted diagram shows the average Spearman's ρ of all correlations within the same region or within different regions.

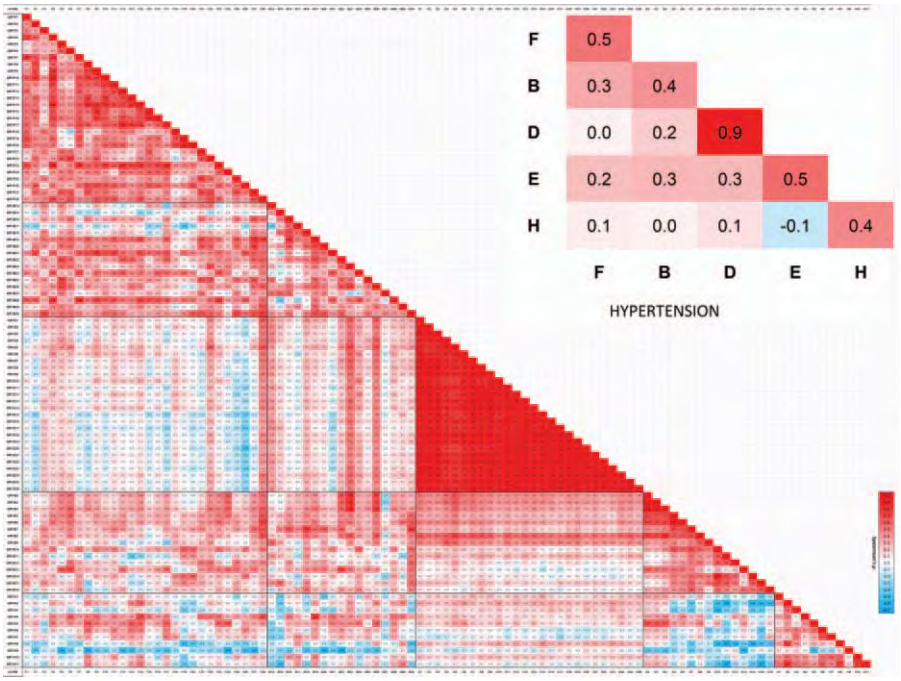

FIGURE 6 Correlation matrix of glucocorticoid receptor gene promoter region DNA methylation (Spearman's ρ shown as heat map). Correlation coefficients of intraregional and interregional glucocorticoid receptor gene proximal promoter CpG site methylation *in hypertensive mothers* (red indicates positive, blue indicates negative correlations). The inserted diagram shows the average Spearman's ρ of all correlations within the same region or within different regions.

numerically reduced ($P = 0.06$) umbilical cord blood levels of cortisol. There is only one study available that presents data regarding associations between maternal hypertension and *NR3C1* 1F promoter methylation. Mansell *et al.* investigated the influence of maternal well being during pregnancy on a wide range of *NR3C1* 1F promoter regions. They demonstrated that maternal hypertension was associated with increased methylation of 1F CpG site 14 [37]. There are several more studies that demonstrated increased promoter 1F methylation in infants exposed to maternal

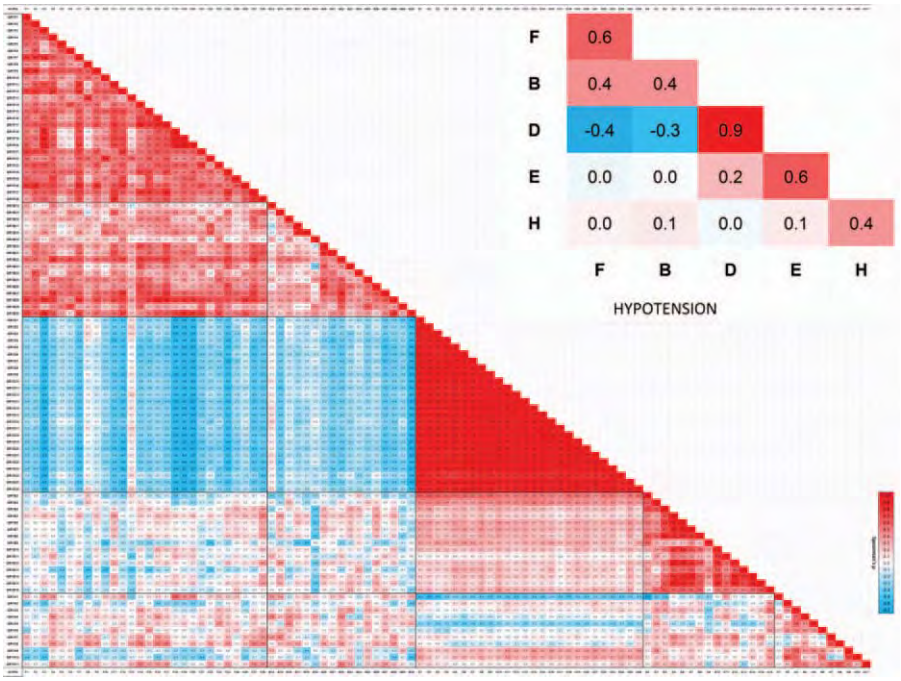

FIGURE 7 Correlation matrix of glucocorticoid receptor gene promoter region DNA methylation (Spearman's ρ shown as heat map). Correlation coefficients of intraregional and interregional glucocorticoid receptor gene proximal promoter CpG site methylation *in hypotensive mothers* (red indicates positive, blue indicates negative correlations). The inserted diagram shows the average Spearman's ρ of all correlations within the same region or within different regions.

stressors during pregnancy, yet no information can be found in regards to maternal BP [38–40]. Considering that anxiety and also depression are associated with an increased risk of hypertension [41–43], it cannot be ruled out that increased maternal BP levels might have been involved in the observed observations.

To assess whether maternal BP is also associated with altered placental expression levels of glucocorticoid receptor, we performed western blots. There were no significant differences in glucocorticoid receptor expression among the three BP groups. Furthermore, there was no significant correlation between the degree of methylation in exon 1F promoter regions and glucocorticoid receptor expression. However, these findings are in accordance with the limited amount of literature available. Until now, there is no evidence that methylation of a single CpG site has functional consequences on glucocorticoid receptor expression [36]. The structure of *NR3C1* is complex, composed of an abundance of alternative first exons, which were shown to be modulated by epigenetic methylation of their promoters, yet the exact impact of DNA methylation on glucocorticoid receptor expression remains incompletely understood [44]. DNA methylation was shown to mark alternative intragenic promoters [45], control alternative splicing [46,47] and even promote gene transcription [48,49]. Current literature suggests that DNA methylation of *NR3C1* promoter regions does not only control relative promoter activity, and levels of individual first exon transcripts, but also impacts on the final protein isoform and its cellular localization [44,50]. There is also considerate evidence that microRNAs are involved in regulating glucocorticoid receptor expression and that glucocorticoid receptor is posttranslationally modified [50]. Alterations in *NR3C1* promoter methylation that were not associated with differences in glucocorticoid receptor expression were also observed in the study by Hogg *et al.* that investigated *NR3C1* promoter methylation in preeclampsia placentae using the Illumina450k array. The authors demonstrated significantly increased DNA methylation in *NR3C1* exon 1D promoter regions in early onset preeclampsia placental samples, which were not correlated with an altered placental glucocorticoid receptor expression (gene expression array data) [51]. Caused by the complex structure of *NR3C1*, there are numerous types of glucocorticoid receptors (GRα, GRβ, GRγ, GR A and GR P) which themselves consist of several isoforms (eight each for GRα and GRβ) [12]. Presumably, absent differences of overall glucocorticoid receptor expression in regards to BP, could have been caused by an opposed regulation of different, yet concomitantly detected glucocorticoid receptor variants (e.g.: GRα: 94 kDa; GRβ: 91 kDa) [12], which could result in a net unchanged level of expression. Such shifts in the expression pattern of glucocorticoid receptor isoforms in the placenta which do not coincide with an overall difference in expression levels have already been demonstrated in preterm birth pregnancies [52].

Taken together, the complex nature of glucocorticoid receptor expression could be an underlying reason why we did not observe any correlation between *NR3C1* 1F methylation and glucocorticoid receptor protein expression.

As it was demonstrated that DNA methylation of *NR3C1* promoters might participate in the complex regulation of glucocorticoid receptor subtype expression [44,50], and also shows a clustered coregulation over short distances [36], we generated correlation matrices, correlating all measured CpG sites and plotted the resulting Spearman's ρs in the form of heatmaps separately for each BP group. Results demonstrated that there is a strong intraregional correlation between CpG site DNA methylation within the GR-1D promoter, irrespective of maternal BP. GR-1E also displayed a strong intraregional correlation in the normotensive group which was less accentuated in the other groups. Significantly, interregional correlation of DNA methylation displayed clustered negative correlations between GR-1B and GR-1D CpG site methylation and between GR-1D and GR-1F CpG site methylation, which were absent in normotensive or hypertensive mothers. Given the u-shaped association between maternal BP and GR-1F methylation, a different impact of GR-1F methylation on interregional methylation patterns could potentially result in different expression patterns of glucocorticoid receptor, resulting in a diverging functional outcome. Although not statistically significant, GR-1D and GR-1B methylation displayed different degrees of CpG site methylation among the three BP groups (Supplemental Fig. 3, http://links.lww.com/HJH/A800). As mentioned before, differences in *NR3C1* methylation patterns can be triggered by glucocorticoid receptor sensitivity [35], which could be one explanation of the observed differences in correlation between *NR3C1* promoters in hypotensive mothers.

Due to the fact that the placentas were obtained after birth, our study design is not able to decide whether the observed alterations in *NR3C1* gene promoter methylation are a cause or a consequence of the pregnancy-related alterations of BP. However, current literature suggests that placental glucocorticoid signaling could be involved in maternal BP regulation during pregnancy. It was demonstrated in associative clinical studies that HDPs are associated with increased placental glucocorticoid metabolism [7]. Moreover, it was shown that increased maternal glucocorticoid levels induce a reduction in placental expression of vascular endothelial growth factor and its receptors together with impaired angiogenesis [53]. It has been extensively demonstrated that reduced placental perfusion can cause widespread dysfunction of the maternal vascular endothelium, by distinct mechanisms that are not fully understood [54,55]. To better understand the relationship between placental DNA methylation of *NR3C1*, placental glucocorticoid signaling and maternal BP regulation, adequately designed animal experiments, analyzing placentas from different stages of pregnancy, are needed.

A limitation of our study is the usage of placental tissue without focusing on a specific placental cell type. We did not do this, as the purification process on its own – as environmental stimuli – may affect epigenetic alterations of the DNA [56]. Support for a potentially neglectable bias of analyzing whole placenta in DNA methylation studies comes from studies that compared epigenetic profiles of isolated trophoblast cells with whole placental tissue. It was shown by MethylC-seq analysis that global methylation in whole rhesus (Rh) placental tissue was almost identical as compared with isolated Rh trophoblast cell methylation

[57]. Furthermore, Grigoriu *et al*. [58], who investigated cell-specific epigenetic differences between isolated trophoblast cells and fibroblasts using the Illumina450k array, also did not find any differences in regards to cell type specifity of *NR3C1* methylation.

In summary, this study provides evidence of an independent association between *NR3C1* proximal promoter methylation and maternal BP regulation during human pregnancy. Our study provided for the first time evidence that for different patterns of placental *NR3C1* promoter methylation in normotensive, hypertensive and hypotensive pregnancy.

Adequately designed animal studies are necessary to address the question whether the observed alterations are causal in the pathogenesis of BP regulation during pregnancy or a consequence of the altered blood pressure.

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Conflicts of interest

There are no conflicts of interest.

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Reviewer's Summary Evaluation

Reviewer 2

Epigenetic mechanisms are important factors in regulation of gene expression. In complex traits such as hypertension, epigenetic factors can reflect some of the interaction between genes and environment. The present study looked into DNA methylation patterns in normotensive, hypertensive and hypotensive pregnancy focusing on the glucocorticoid receptor gene promoter in human placenta. The authors describe characteristic patterns for these three conditions that do, however, not translate into different gene expression. Despite a number of methodological issues that are appropriately discussed in the paper, the authors provide important descriptive data that pave the way for future mechanistic studies. The paper certainly reminds us yet again of the complexity of glucocorticoid signalling in pregnancy and in general.


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Original Article

DNA methylation of the glucocorticoid receptor gene promoter in the placenta is associated with blood pressure regulation in human pregnancy

Sulistyo E. Dwi Putra[a,b,*], Christoph Reichetzeder[b,c,*], Martin Meixner[d], Karsten Liere[d], Torsten Slowinski[e], and Berthold Hocher[b,f,g]

Background: Blood pressure (BP) regulation during pregnancy is influenced by hormones of placental origin. It was shown that the glucocorticoid system is altered in hypertensive pregnancy disorders such as preeclampsia. Epigenetic mechanism might influence the activity of genes involved in placental hormone/hormone receptor synthesis/action during pregnancy.

Method: In the current study, we analyzed the association of 5′-C-phosphate-G-3′ (CpG) site methylation of different glucocorticoid receptor gene (*NR3C1*) promoter regions with BP during pregnancy. The study was performed as a nested case-control study ($n=80$) out of 1045 mother/child pairs from the Berlin Birth Cohort. Placental DNA was extracted and bisulfite converted. Nested PCR products from six *NR3C1* proximal promoter regions [glucocorticoid receptor gene promotor region B (GR-1B), C (GR-1C), D (GR-1D), E (GR-1E), F (GR-1F), and H (GR-1H)] were analyzed by next generation sequencing.

Results: NR3C1 promoter regions GR-1D and GR-1E had a much higher degree of DNA methylation as compared to GR-1B, GR-1F or GR-1H when analyzing the entire study population. Comparison of placental *NR3C1* CpG site methylation among hypotensive, normotensive and hypertensive mothers revealed several differently methylated CpG sites in the GR-1F promoter region only. Both hypertension and hypotension were associated with increased DNA methylation of GR-1F CpG sites. These associations were independent of confounding factors, such as family history of hypertension, smoking status before pregnancy and prepregnancy BMI. Assessment of placental glucocorticoid receptor expression by western blot showed that observed DNA methylation differences were not associated with altered levels of placental glucocorticoid receptor expression. However, correlation matrices of all *NR3C1* proximal promoter regions demonstrated different correlation patterns of intraregional and interregional DNA methylation in the three BP groups, putatively indicating altered transcriptional control of glucocorticoid receptor isoforms.

Conclusion: Our study provides evidence of an independent association between placental *NR3C1* proximal promoter methylation and maternal BP. Furthermore, we observed different patterns of *NR3C1* promoter methylation in normotensive, hypertensive and hypotensive pregnancy.

Keywords: DNA methylation, epigenetics, glucocorticoid receptor, hypertension, hypotension, *NR3C1* gene, placenta, pregnancy

Abbreviations: GR, glucocorticoid receptor; GR-1B, glucocorticoid receptor gene promotor region B; GR-1C, glucocorticoid receptor gene promotor region C; GR-1D, glucocorticoid receptor gene promotor region D; GR-1E, glucocorticoid receptor gene promotor region E; GR-1F, glucocorticoid receptor gene promotor region F; GR-1H, glucocorticoid receptor gene promotor region H; MAP, mean arterial blood pressure; *NR3C1*, glucocorticoid receptor gene

INTRODUCTION

Hypertensive disorders of pregnancy (HDP) are a common pregnancy complication. HDP encompass several diseases, including chronic hypertension, gestational hypertension, preeclampsia and eclampsia [1]. According to the WHO, the prevalence of HDP is as high as 14% [2]. HDP is associated with adverse pregnancy outcomes for both, mother and fetus [3]. On the other hand, hypotension during pregnancy is also associated with impaired pregnancy outcomes [4].

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Blood pressure (BP) regulation during pregnancy is mediated at least partially by hormonal factors [5]. A crucial hormone in this regard is cortisol [6]. The production of cortisol increases several times during pregnancy [6]. It was reported that cortisol catabolism is more pronounced in HDP compared with normal pregnancies [7]. In particular cortisol metabolism in the human placenta is linked to HDP in humans [8]. Our knowledge of a putative association between cortisol metabolism and hypotension during pregnancy, however, is limited so far. Cortisol likewise plays a key role in organ development of the growing fetus. This seems to be in particular important for the development of the human brain [9].

Cortisol effects are mediated by the glucocorticoid receptor. After binding cortisol, the cortisol–glucocorticoid receptor complex has the ability to bind glucocorticoid response elements in the promoter region of cortisol responsive genes. Together with transcription factors, this cascade leads to an increased or decreased gene expression of – for example – genes involved in BP regulation [10]. Henceforth, in addition to circulating cortisol levels, the degree of glucocorticoid receptor expression is an important factor in determining the effect of cortisol on BP [11]. The glucocorticoid receptor is expressed in the placenta [12]. The interaction between placental glucocorticoid receptor and cortisol affects placental growth and development [13], and supports the central placental function as a transfer organ between mother and fetus [14]. Impaired glucocorticoid receptor expression in the placenta can affect fetal growth and development [13]. Current literature furthermore suggests an involvement of glucocorticoid receptor expression in preeclampsia [15]. Modulation of gene expression in the placenta may be mediated by epigenetic mechanisms, including DNA methylation [16,17]. It was reported that BP is correlated with DNA methylation patterns in placental genes [18]. Specifically for the glucocorticoid receptor gene (*NR3C1*), it had been reported that stress during pregnancy can influence the methylation level of this gene [19]. However, there is no study yet that investigated if there is a correlation between maternal BP as another parameter determining the prenatal environment and the methylation level of *NR3C1*.

NR3C1 contains two distinguished groups of promoters termed distal and proximal promoters [20]. The group of proximal promoters consists of several different promoter regions, including glucocorticoid receptor gene promotor region D (GR-1D), glucocorticoid receptor gene promotor region E (GR-1E), glucocorticoid receptor gene promotor region B (GR-1B), glucocorticoid receptor gene promotor region F (GR-1F), glucocorticoid receptor gene promotor region C (GR-1C) and glucocorticoid receptor gene promotor region H (GR-1H) [21]. Proximal promoter regions are potential targets for epigenetic modifications, as they are located in a 5′-C-phosphate-G-3′ (CpG) island [21]. There is growing evidence that CpG island promoter methylation is important for the modulation of gene expression [22]. Moreover, the methylation level of proximal promoters is sensitive to factors determining the prenatal environment, including maternal BP [21] The purpose of this study was to explore the correlation between maternal BP and the methylation level of placental *NR3C1* proximal promoter regions and to identify potential patterns of glucocorticoid receptor promoter methylation that are specific for hypertensive or hypotensive pregnancies.

MATERIALS AND METHODS

Clinical study

This nested case–control study is a part of Berlin Birth Cohort study [23]. The study was approved by the Institutional Review Board of the university hospital of Charité, Berlin, Germany [24,25]. The study was conducted in 1045 placental samples from mothers [26] who delivered in the obstetrics department of Campus Charité Mitte, Berlin, Germany. Structured interviews were carried out after obtaining written consent to take part in the study. A pregnancy health document (Mutterpass) was used to extract relevant data such as diabetes history, hypertension history and smoking status. Gestational age was calculated based on the last menstruation period. Routine antenatal examinations were used to collect SBP and DBP data. For all individuals, mean SBP and DBP were calculated from all available readings. Eighty placenta samples were chosen as a representative of 1045 placenta samples, considering SBP and DBP of the mother in the 3rd trimester of pregnancy. Biometric parameters such as sex of the newborn, birth size and APGAR score were documented during the postnatal examination. A standardized placenta sample (one cotyledon from similar locations) was obtained, immediately frozen and stored at −20 °C. A sample of chorionic villi was acquired from the cotyledon samples for DNA and protein extraction.

DNA isolation and bisulfit treatment

DNA extraction was performed using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the recommended procedure by the manufacturer. RNA interference was minimalized by adding RNAse during the DNA isolation process. DNA solution was then analyzed with a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific, Renfrew, UK) to measure quality and quantity of the DNA. DNA and protein content were measured as absorbance at 260 and 280 nm, respectively. Only samples with 260/280 nm absorbance ratio between 1.9 and 2.1 were considered to be processed further. Bisulfite treatment was performed using EZ-96 DNA methylation kit following the protocol from the manufacturer for an amount of maximal 500-ng DNA in each sample solution.

PCR and illumina sequencing

Nested PCR was performed to prepare the amplicons for sequencing on an Illumina MiSeq. PCR primers were designed based on the selected areas of *NR3C1* which included the proximal promoters GR-1D, GR-1E, GR-1B, GR-1F, GR-1C and GR-1H (Fig. 1; Supplemental Table 1, http://links.lww.com/HJH/A800). The first PCR was conducted using an outer primer (Supplemental Table 1, http://links.lww.com/HJH/A800) by applying the following protocol: predenaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, annealing at approximate melting temperature of the outer promoter for 30 s (Supplemental

FIGURE 1 Schematic diagram of selected glucocorticoid receptor gene CpG sites which were explored in this study. Five PCR products from six different glucocorticoid receptor gene proximal promoter areas were designed in this study. The corresponding sequences for designed primers are indicated in bold characters. Numbers in the box for every PCR product indicate the relative position of the last base in the PCR product to the transcription-starting site. Glucocorticoid receptor gene promotor region D, glucocorticoid receptor gene promotor region E, glucocorticoid receptor gene promotor region B/1F, glucocorticoid receptor gene promotor region F/glucocorticoid receptor gene promotor region C and glucocorticoid receptor gene promotor region H are primers for amplification of glucocorticoid receptor gene promotor region D, glucocorticoid receptor gene promotor region E, glucocorticoid receptor gene promotor region B/1F, glucocorticoid receptor gene promotor region F/1C and glucocorticoid receptor gene promotor region H of the glucocorticoid receptor gene proximal promoter, respectively. Forward (Fwd) or reverse (Rev) primers are indicated. The first translation site is located in the exon-2 (arrow above the box). The numbering of CpG sites was assigned based on a previous study.

Table 1, http://links.lww.com/HJH/A800) and 72 °C for 4 min for extension step. Final extension was performed at 72 °C for 10 min. The obtained amplicons were further used as templates for a second PCR using inner primer (Supplemental Table 1, http://links.lww.com/HJH/A800). The inner primers are completed with 10 individual barcodes for the sequencing purpose. Ten barcodes which were used were TCGCAGG, CTCTGCA, CCTAGGT, GGATCAA, GCAAGAT, ATGGAGA, CTCGATG, GCTCGAA, ACCAACT and CCGGTAC. Five different nested PCR products from six *NR3C1* proximal promoter areas were designed in this study (Fig. 1). After adjusting the concentration of the nested PCR products to equal amounts using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, California, USA), amplicon pools were prepared for sequencing with AMPure XP beads (Beckman Coulter Genomics, Danvers, Massachusetts, USA). Size and quantity of the amplicon library were assessed on a Fragment Analyzer (AATI; Analytical Technologies, Inc., Ankeny, Iowa, USA). The libraries were clustered to a density of approximately 800 K/mm^2 and sequenced with 2× 300v3 paired-end MiSeq runs. Image analysis, base calling and data quality assessment were performed on the MiSeq instrument.

Data processing

The sequence reads were merged, trimmed and demultiplexed using CLC Genomics Workbench 7 software (Qiagen) using the default parameters with 200 bp as minimum length. Subsequently, at least 20 processed reads were analyzed for their DNA methylation level quantification using the BiQ Analyzer HT software (Max-Planck-Institut for Informatics, Computational Biology and Applied Algorithmics, Germany) [27].

Western blots

Western blots were performed as previously described [28]. Chorionic villi samples were pulverized in a metal mortar after cooling in liquid nitrogen. Protein extraction was performed using a urea/thiourea buffer [2 mol/l thiourea, 7 mol/l urea, 2% SDS, 1% dithiothreitol and protease inhibitor (Complete Mini, Cat. No.: 11 697 498 001; Roche)]. Protein extracts were separated by SDS–PAGE employing a 10% polyacrylamide gel. After electrophoresis, gels were blotted onto nitrocellulose membranes (AmershamTM HybondTM ECL; GE Healthcare, Little Chalfont, UK) using a Biorad Trans-Blot semidry blotter and transfer buffer (184 mmol/l glycine, 24 mmol/l Tris, 20% methanol). The primary antibody incubation (anti-glucocorticoid receptor, sc-8992; Santa Cruz Biotechnology, Dallas, Texas, USA) was performed at 4 °C overnight. After washing with TBS-T, the membrane was incubated in secondary antibody (horseradish-peroxidase conjugated antigoat IgG; sc-2020; Santa Cruz Biotechnology) for 1 h at room temperature. Protein signals were developed using enhanced chemiluminescence solution. As a housekeeping protein expression of glyceraldehyde 3-phospate dehydrogenase (GAPDH) was used (anti-GAPDH, sc-32233; Santa Cruz Biotechnology). The GAPDH signal was developed using an alkaline phosphatase-conjugated secondary antibody (antimouse IgG,

TABLE 1. Characteristics of the explored mother–child pairs data

Parameter	Hypotension (27)	Normotension (33)	Hypertension (20)	*P*
Gestational age at delivery (week)	39.0 ± 1.8	38.9 ± 1.4	38.3 ± 2.0	0.290
Age of the mother (year)	30.7 ± 7.6	29.1 ± 5.2	30.6 ± 4.6	0.524
Prepregnancy BMI (kg/m^2)	21.0 ± 3.0	23.6 ± 4.3	28.7 ± 6.5	<0.0001
SBP 3rd trimester (mmHg)	96.8 ± 9.2	118.6 ± 13.8	144.9 ± 7.7	<0.0001
DBP 3rd trimester (mmHg)	55.0 ± 3.8	72.6 ± 9.3	89.9 ± 4.2	<0.0001
Smoking before pregnancy (%)	25.9	45.5	45.0	0.244
Hypertension in family (%)	33.3	39.4	80.0	0.003
Diabetes in family (%)	26.1	26.7	61.5	0.055
Ethnicity, white/nonwhite	88.9	93.9	100.0	0.298
Child birth weight (g)	3342.6 ± 636.5	3249.1 ± 392.5	3098.8 ± 649.9	0.331
Child head circumference (cm)	34.6 ± 1.7	34.6 ± 1.3	33.8 ± 1.7	0.212
Child birth length (cm)	50.8 ± 3.4	50.0 ± 3.4	49.4 ± 2.8	0.313
Ponderalindex	25.3 ± 2.2	26.9 ± 9.9	25.4 ± 2.0	0.586
APGAR score 5 min	9.3 ± 1.0	9.3 ± 1.3	8.6 ± 2.1	0.296
APGAR score 10 min	9.8 ± 0.5	9.6 ± 0.7	9.2 ± 1.4	0.114
Sex of the child, male/female	44.4/55.6	45.5/54.5	40.0/60.0	0.924

Data are given as mean ± SD or %.

sc-2008; Santa Cruz Biotechnology) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride (BCIP/NBT) as substrate. The developed membranes were digitalized using a 600 dpi scanner resolution and analyzed with AlphaEaseFCTM (Alpha Innotech, San Leandro, California, USA).

Statistical analysis

Statistical analyses were performed using SPSS version 20 (SPSS Inc., Chicago, IL, USA). BP grouping was performed based on previously published criteria [29,30]. Gestational hypertension was defined as mothers who had mean SBP at least 140 or DBP at least 90 mmHg in the second half of pregnancy, but no history of hypertension prior to pregnancy. The control group accommodated mothers who had SBP between 90 and 140 and DBP between 60 and 90 mmHg. Hypotension was defined as SBP less than 90 or DBP less than 60 mmHg in the second half of pregnancy, but no history of hypotension prior to pregnancy (for details regarding distribution and cutoffs of SBP and DBP see Supplemental Fig. 1, http://links.lww.com/HJH/A800). Unpaired *t* test or Mann–Whitney *U* test was carried out to compare continuous parameters, if two groups were compared. Normal distribution of the data was tested using the Kolmogorov–Smirnov test. Based on normal distribution of the data analysis of variance (ANOVA) analysis followed by a Tukey honestly significant difference (HSD) post-hoc test or Kruskal–Wallis followed by Dunn–Bonferroni post-hoc test was used to compare continuous parameters between the three BP groups. Comparisons of categorical parameters among different BP groups were performed using chi-squared test. Independent association analysis between CpG site methylation and BP groups was performed by using multinominal logistic regression models adjusted for established factors influencing BP, that were found to be significantly correlated to DBP or SBP in the analyzed cohort (Supplemental Table 3, http://links.lww.com/HJH/A800). Bar graph calculation and compilation was performed using Graphpad Prism 5 (GraphPad Software Inc., La Jolla, California, USA).

RESULTS

Comparison analyses demonstrated no statistically significant differences in regards to clinically important parameters between the sample population ($n = 1045$) and the 80 selected samples of the representative cohort (Supplemental Table 2, http://links.lww.com/HJH/A800). These results indicate that the selected 80 samples can be used as a representation of the whole sample population. Table 1 displays detailed descriptive statistics of the 80 selected mothers and their newborns. The three BP groups consisted of 27 hypotensive, 33 normotensive and 20 hypertensive mothers (Table 1). ANOVA analysis followed by a Tukey HSD post-hoc test statistically substantiated BP differences in the 3rd trimester of pregnancy between the three groups [SBP ($P < 0.0001$) and DBP ($P < 0.0001$)]. Prepregnancy BMI was also significantly ($P < 0.0001$) different among the BP groups. No parameter of the newborns was found to be different among the three BP groups.

Figure 2 displays the degree of DNA methylation among all CpG sites analyzed in this study. Generally, GR-1D and GR-1E showed more DNA methylation compared with GR-1B, GR-1F or GR-1H. DNA methylation of GR-1C could not be analyzed in this study because of a too small number of reads in the next generation sequencing analysis. Comparison of the CpG site methylation level revealed several differently methylated CpG sites of GR-1F among the three BP groups. However, no significant differences were observed at any of the other studied glucocorticoid receptor CpG sites. Comparison of GR-1F CpG site methylation levels among the three BP groups showed a significantly higher DNA methylation of GR-1F CpG sites 1, 2, 12, 16, 20, 24 and 26 in placentas from hypertensive mothers compared with normotensive mothers (Fig. 3). Moreover, the levels of DNA methylation of GR-1F CpG sites 1, 3, 12, 12 and 20 were significantly higher in hypotensive compared with normotensive mothers.

To demonstrate that DNA methylation of GR-1F is independently associated with BP, multinominal logistic regression analyses were performed and adjusted for well known confounders impacting on BP that were

TABLE 2. Multinominal logistic regression analysis of the association between CpG site methylation in glucocorticoid receptor gene promotor region F and blood pressure group (dependent variable)

Methylation at CpG position	Blood pressure group	B	SE	*P*	Exp (B)	95% CI for exp (B)	
						Min	Max
1	Hypotension[a]	0.33	0.20	0.092	1.39	0.95	2.05
	Hypertension[a]	0.44	0.20	**0.029**	1.55	1.05	2.29
2	Hypotension[a]	0.22	0.15	0.140	1.25	0.93	1.69
	Hypertension[a]	0.42	0.16	**0.010**	1.52	1.11	2.10
3	Hypotension[a]	0.24	0.09	**0.006**	1.27	1.07	1.51
	Hypertension[a]	0.07	0.10	0.488	1.07	0.89	1.29
12	Hypotension[a]	0.37	0.18	**0.040**	1.44	1.02	2.05
	Hypertension[a]	0.62	0.20	**0.002**	1.86	1.26	2.73
16	Hypotension[a]	0.36	0.22	0.095	1.43	0.94	2.19
	Hypertension[a]	0.74	0.25	**0.003**	2.10	1.29	3.42
20	Hypotension[a]	0.18	0.09	**0.046**	1.19	1.00	1.42
	Hypertension[a]	0.26	0.09	**0.005**	1.30	1.08	1.55
24	Hypotension[a]	0.12	0.15	0.453	1.12	0.83	1.52
	Hypertension[a]	0.46	0.18	**0.012**	1.58	1.11	2.25
26	Hypotension[a]	0.07	0.13	0.570	1.08	0.83	1.39
	Hypertension[a]	0.32	0.15	**0.026**	1.38	1.04	1.83

CI, confidence interval.
[a]Normotension was set as reference for these parameters. The models were confounded for: history of hypertension, age of the mother and prepregnancy BMI. Bold values indicate $P < 0.05$.

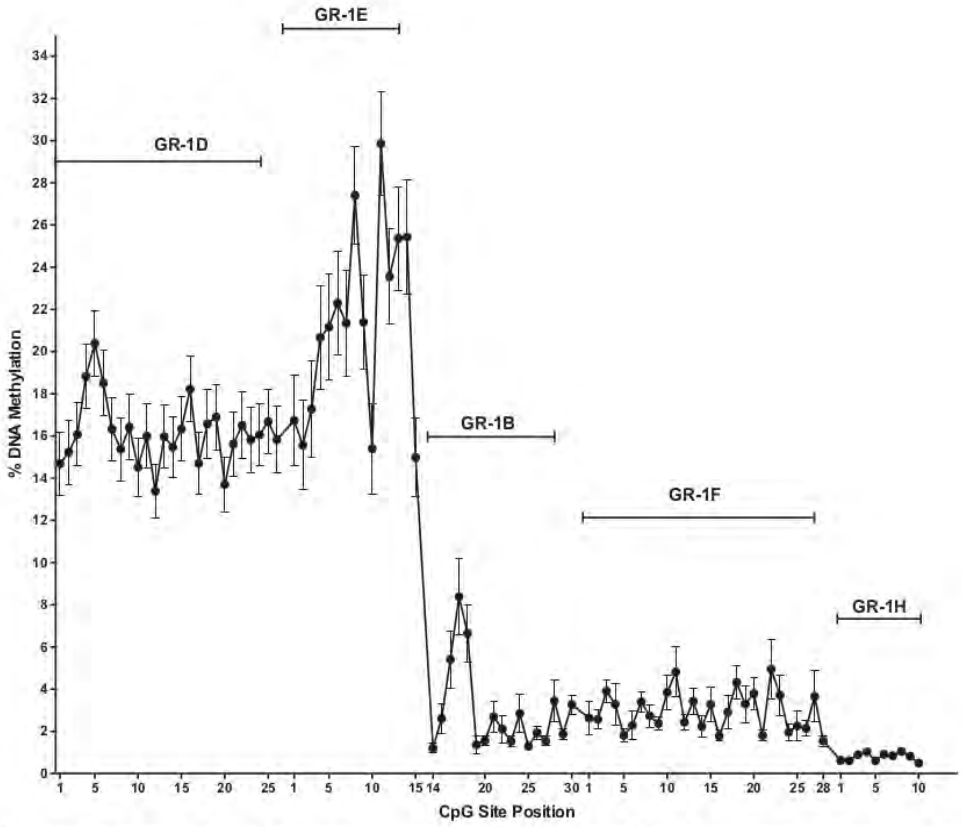

FIGURE 2 Methylation level of several CpG sites in five glucocorticoid receptor gene proximal promoters area. Different proximal promoters of glucocorticoid receptor gene showed different average methylation levels. Error bars represent SEMs. Graphs represent mean ± SEM.

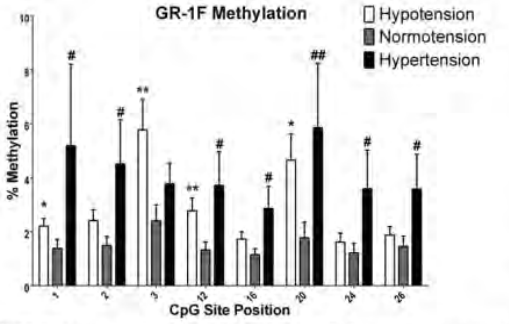

FIGURE 3 Mean methylation comparison of several CpG sites on glucocorticoid receptor gene promotor region F among blood pressure groups in univariate analysis. Only CpG sites with significantly different mean methylation level were shown. # and ## indicate comparison between normotension and hypertension with $P < 0.05$ and $P < 0.01$, respectively. * and ** indicate comparison between normotension and hypotension with $P < 0.05$ and $P < 0.01$, respectively. Error bars represent SEMs.

significantly correlated to DBP or SBP in the analyzed cohort (history of hypertension, smoking before pregnancyand prepregnancy BMI; Supplemental Table 3, http://links.lww.com/HJH/A800). Family history of diabetes was also correlated to DBP and SBP. As 14 values were missing in this variable and results were not altered by its inclusion to the model (data not shown), we did not include the variable to the model. Comparing CpG site methylation between the hypertensive and the normotensive group demonstrated an independent positive association of GR-1F CpG sites 1 ($P < 0.05$), 2 ($P < 0.05$), 12 ($P < 0.01$), 16 ($P < 0.01$), 20 ($P < 0.01$), 24 ($P < 0.05$) and 26 ($P < 0.05$) with hypertension. Compared with the normotensive group, the hypotensive group was independently associated with increased methylation of GR-1F CpG sites, 3 ($P < 0.01$), 12 ($P < 0.01$) and 20 ($P < 0.05$) (Table 2).

To assess if placental glucocorticoid receptor expression is affected by the observed increases in GR-1F CpG site methylation in hypotensive and hypertensive mothers, Western blots were performed. There were no significant differences in the level of placental glucocorticoid receptor expression among the three BP groups (Fig. 4). Furthermore, there was no significant correlation between GR-1F CpG sites and placental glucocorticoid receptor expression (data not shown). To investigate whether the degree of methylation of a given individual CpG site is correlated with other CpG sites within the same and also within different proximal promoter regions, spearman correlation matrices were calculated for each BP group. Resulting Spearman's ρs were plotted as heat maps separately for each BP group (Figs. 5–7) and average correlation coefficients for all possible regional interactions were calculated (see inserted diagram in each figure). Strong intraregional correlations were found in all three BP groups for GR-1D methylation (average spearman's $\rho = 0.9$) in all three groups. GR-1E methylation also displayed a strong intraregional correlation in the normotensive group (spearman's $\rho = 0.8$) which was less accentuated in the hypotensive (spearman's $\rho = 0.6$) and the hypertensive (spearman's $\rho = 0.5$) group. A more moderate intraregional correlation was observed for GR-1F methylation (spearman's $\rho = 0.5–0.6$ in all groups). In regards to interregional correlation of *NR3C1* proximal promoters, different patterns were observed in the three BP groups. Although hypotensive mothers displayed moderate (average spearman's ρ of the whole regions) to strong (correlations of clusters within promoter regions) negative correlations between GR-1B and GR-1D (average spearman's $\rho = -0.3$), and between GR-1D and GR-1F (average spearman's $\rho = -0.4$), these correlation patterns were not observed in normotensive or hypertensive mothers.

DISCUSSION

We provide evidence of an independent association between placental CpG island methylation pattern of the *NR3C1* promoter region GR-1F and BP regulation during pregnancy. Our findings are in agreement with data coming from nonpregnant hypertension studies showing that BP regulation can be influenced by *NR3C1* expression [31]. Moreover, our study provides evidence for different patterns of placental *NR3C1* promoter methylation in normotensive, hypertensive and hypotensive pregnancy.

The current study is the first to evaluate a potential association of maternal BP during pregnancy and methylation of *NR3C1* proximal promoter CpG sites. It is important to note that the findings of our study are independent of known confounding factors/risk factors of hypertensive disorders during pregnancy, such as family history of hypertension, smoking status and BMI (Table 2).

The result of this study reinforces the impact of the GR-1F methylation region in association with environmental factors as it has been reviewed previously [21].

However, we did not see a correlation between GR-1D promoter region methylation and BP regulation during pregnancy as it was seen by Hogg *et al.* [15]. There are several reasons that might explain this. Hogg *et al.* [15] focused on the comparison of healthy pregnant women and preeclamptic women. We on the other hand compared pregnant women with normal BP with pregnant women developing high BP during pregnancy independently from the degree of urinary protein excretion. The ethnic background of the study population analyzed by Hogg *et al.*, [15] analyzed at the University of British Columbia, Vancouver, British Columbia, Canada did display differences to the current study in regards to a larger proportion of Asian women (30%). Moreover, feeding habits of pregnant women in Berlin and Vancouver might also be different. It is for example very likely that the vitamin D status differed in both cohorts. Vitamin D during pregnancy, however, has a huge impact on pregnancy outcome [32]. Moreover, there are methodical differences that might also contribute at least partially to the differences seen in both studies. We analyzed six *NR3C1* proximal promoter regions (GR-1D, GR-1E, GR-1B/GR-1F, GR-1F/GR-1C and GR-1H) by next generation sequencing, whereas Hogg *et al.* [15] studied DNA methylation by bisulfite pyrosequencing.

A novelty of the current study is the inclusion of mothers experiencing hypotension during pregnancy. Interestingly the association between maternal BP and GR-1F methylation displayed a u-shaped behavior, with increased GR-1F methylation found in both mothers with hypotension and

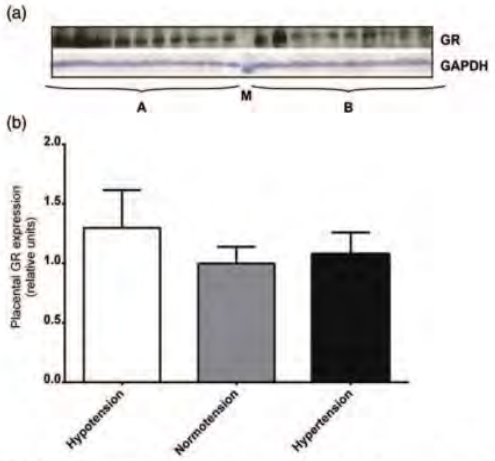

FIGURE 4 (a) Representative picture of glucocorticoid receptor western blot result and its corresponding housekeeping gene. On the left side (A), five different amounts of placental protein standard solution were pipette in two replicates, then a molecular weight marker (M), followed by individual protein samples as well in two replicates (B). Molecular weights were measured at 94 kDa for glucocorticoid receptor and at 37 kDa for glyceraldehyde 3-phospate dehydrogenase. (b) Placental expression of the glucocorticoid receptor among hypotensive, normotensive and hypertensive mothers. There were no significant differences in placental glucocorticoid receptor expression among the three blood pressure groups. Data shown ± SEM. GAPDH, glyceraldehyde 3-phospate dehydrogenase; GR, glucocorticoid receptor.

hypertension. Given a general lack of literature, it is hard to interpret this – at first sight – counterintuitive result. However, especially for glucocorticoid signaling and glucocorticoid receptor function, u-shaped and inverted u-shaped relationships were shown [33]. For example, memory retrieval is impaired, both, at very low as well as very high cortisol levels, but not at intermediate levels [34]. Such u-shaped effects of cortisol might be caused by its function through several receptor subtypes, which bear specific characteristics such as accessibility, affinity, desensitization or triggered signaling cascade [33]. Furthermore, interaction of cortisol with other receptors, such as the mineralcorticoid receptor, and its metabolism by 11-b-hydroxysteroid dehydrogenases might further contribute to u-shaped effects of cortisol [33]. Given the u-shaped behavior of glucocorticoid receptor signaling, it might be possible that methylation processes in *NR3C1* follow comparable patterns. Cell culture experiments in endothelial cells have demonstrated that glucocorticoid receptor signaling impacts on DNA methylation of *NR3C1* promoter sites [35]. Moreover, it was shown that depending on glucocorticoid sensitivity, different patterns of *NR3C1* promoter methylation emerge [35].

Until now, there have been no comparable studies that investigated associations between, both, hypotension and hypertension and *NR3C1* 1F promoter methylation. However, there are several studies that demonstrated associations between lower BP levels and higher methylation. Tempel *et al.* [36] showed in a cohort of healthy individuals that decreased baseline BP was associated with increased overall *NR3C1* 1F promoter methylation levels in DNA extracted from whole blood. Mata-Greenwood *et al.*, who investigated the impact of glucocorticoid receptor signaling on *NR3C1* 1F promoter methylation levels in dexamethasone resistant and dexamethasone sensitive human umbilical vein endothelial cells, demonstrated increased 1F promoter methylation in dexamethasone resistant cells. Significantly, mothers from whom these cells were harvested displayed significantly lower SBP levels and

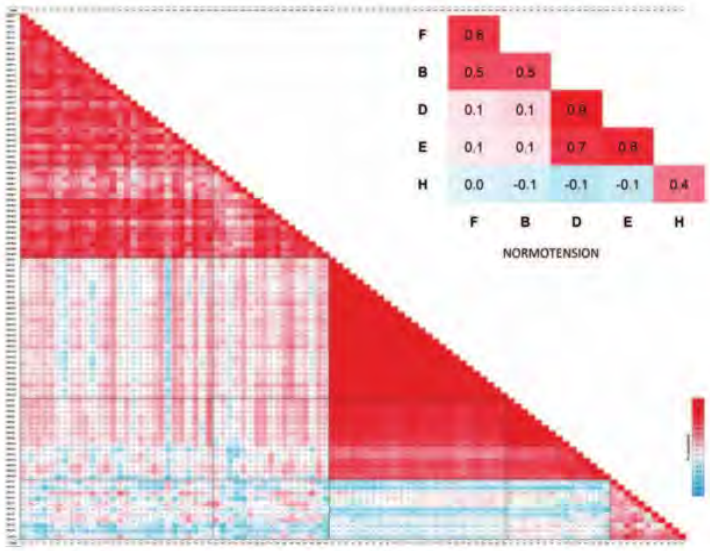

FIGURE 5 Correlation matrix of glucocorticoid receptor gene promoter region DNA methylation (Spearman's ρ shown as heat map). Correlation coefficients of intraregional and interregional glucocorticoid receptor gene proximal promoter CpG site methylation *in normotensive mothers* (red indicates positive, blue indicates negative correlations). The inserted diagram shows the average Spearman's ρ of all correlations within the same region or within different regions.

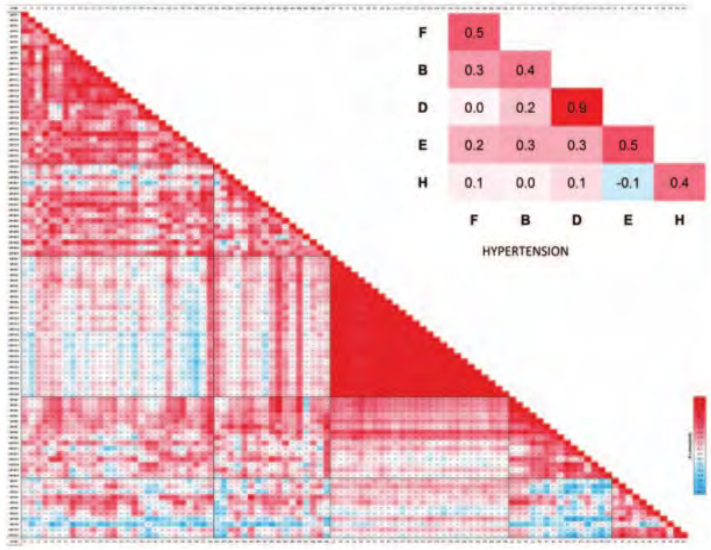

FIGURE 6 Correlation matrix of glucocorticoid receptor gene promoter region DNA methylation (Spearman's ρ shown as heat map). Correlation coefficients of intraregional and interregional glucocorticoid receptor gene proximal promoter CpG site methylation *in hypertensive mothers* (red indicates positive, blue indicates negative correlations). The inserted diagram shows the average Spearman's ρ of all correlations within the same region or within different regions.

numerically reduced ($P = 0.06$) umbilical cord blood levels of cortisol. There is only one study available that presents data regarding associations between maternal hypertension and *NR3C1* 1F promoter methylation. Mansell *et al.* investigated the influence of maternal well being during pregnancy on a wide range of *NR3C1* 1F promoter regions. They demonstrated that maternal hypertension was associated with increased methylation of 1F CpG site 14 [37]. There are several more studies that demonstrated increased promoter 1F methylation in infants exposed to maternal

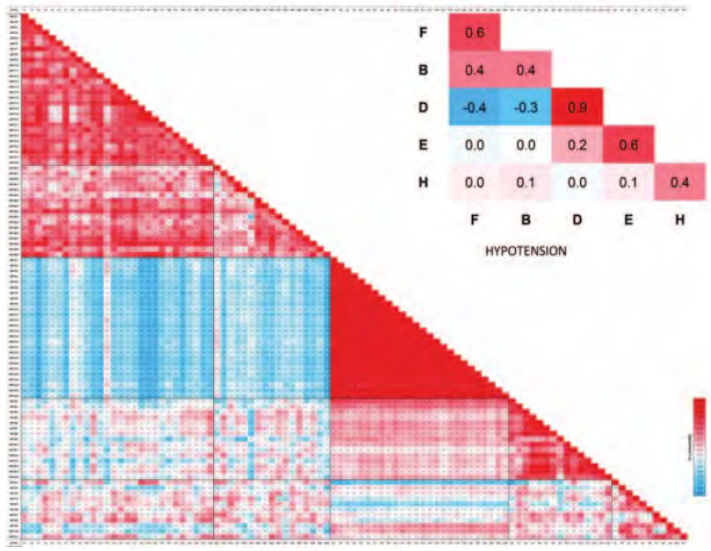

FIGURE 7 Correlation matrix of glucocorticoid receptor gene promoter region DNA methylation (Spearman's ρ shown as heat map). Correlation coefficients of intraregional and interregional glucocorticoid receptor gene proximal promoter CpG site methylation *in hypotensive mothers* (red indicates positive, blue indicates negative correlations). The inserted diagram shows the average Spearman's ρ of all correlations within the same region or within different regions.

stressors during pregnancy, yet no information can be found in regards to maternal BP [38–40]. Considering that anxiety and also depression are associated with an increased risk of hypertension [41–43], it cannot be ruled out that increased maternal BP levels might have been involved in the observed observations.

To assess whether maternal BP is also associated with altered placental expression levels of glucocorticoid receptor, we performed western blots. There were no significant differences in glucocorticoid receptor expression among the three BP groups. Furthermore, there was no significant correlation between the degree of methylation in exon 1F promoter regions and glucocorticoid receptor expression. However, these findings are in accordance with the limited amount of literature available. Until now, there is no evidence that methylation of a single CpG site has functional consequences on glucocorticoid receptor expression [36]. The structure of *NR3C1* is complex, composed of an abundance of alternative first exons, which were shown to be modulated by epigenetic methylation of their promoters, yet the exact impact of DNA methylation on glucocorticoid receptor expression remains incompletely understood [44]. DNA methylation was shown to mark alternative intragenic promoters [45], control alternative splicing [46,47] and even promote gene transcription [48,49]. Current literature suggests that DNA methylation of *NR3C1* promoter regions does not only control relative promoter activity, and levels of individual first exon transcripts, but also impacts on the final protein isoform and its cellular localization [44,50]. There is also considerate evidence that microRNAs are involved in regulating glucocorticoid receptor expression and that glucocorticoid receptor is posttranslationally modified [50]. Alterations in *NR3C1* promoter methylation that were not associated with differences in glucocorticoid receptor expression were also observed in the study by Hogg *et al.* that investigated *NR3C1* promoter methylation in preeclampsia placentae using the Illumina450k array. The authors demonstrated significantly increased DNA methylation in *NR3C1* exon 1D promoter regions in early onset preeclampsia placental samples, which were not correlated with an altered placental glucocorticoid receptor expression (gene expression array data) [51]. Caused by the complex structure of *NR3C1*, there are numerous types of glucocorticoid receptors (GRα, GRβ, GRγ, GR A and GR P) which themselves consist of several isoforms (eight each for GRα and GRβ) [12]. Presumably, absent differences of overall glucocorticoid receptor expression in regards to BP, could have been caused by an opposed regulation of different, yet concomitantly detected glucocorticoid receptor variants (e.g.: GRα: 94 kDa; GRβ: 91 kDa) [12], which could result in a net unchanged level of expression. Such shifts in the expression pattern of glucocorticoid receptor isoforms in the placenta which do not coincide with an overall difference in expression levels have already been demonstrated in preterm birth pregnancies [52].

Taken together, the complex nature of glucocorticoid receptor expression could be an underlying reason why we did not observe any correlation between *NR3C1* 1F methylation and glucocorticoid receptor protein expression.

As it was demonstrated that DNA methylation of *NR3C1* promoters might participate in the complex regulation of glucocorticoid receptor subtype expression [44,50], and also shows a clustered coregulation over short distances [36], we generated correlation matrices, correlating all measured CpG sites and plotted the resulting Spearman's ρs in the form of heatmaps separately for each BP group. Results demonstrated that there is a strong intraregional correlation between CpG site DNA methylation within the GR-1D promoter, irrespective of maternal BP. GR-1E also displayed a strong intraregional correlation in the normotensive group which was less accentuated in the other groups. Significantly, interregional correlation of DNA methylation displayed clustered negative correlations between GR-1B and GR-1D CpG site methylation and between GR-1D and GR-1F CpG site methylation, which were absent in normotensive or hypertensive mothers. Given the u-shaped association between maternal BP and GR-1F methylation, a different impact of GR-1F methylation on interregional methylation patterns could potentially result in different expression patterns of glucocorticoid receptor, resulting in a diverging functional outcome. Although not statistically significant, GR-1D and GR-1B methylation displayed different degrees of CpG site methylation among the three BP groups (Supplemental Fig. 3, http://links.lww.com/HJH/A800). As mentioned before, differences in *NR3C1* methylation patterns can be triggered by glucocorticoid receptor sensitivity [35], which could be one explanation of the observed differences in correlation between *NR3C1* promoters in hypotensive mothers.

Due to the fact that the placentas were obtained after birth, our study design is not able to decide whether the observed alterations in *NR3C1* gene promoter methylation are a cause or a consequence of the pregnancy-related alterations of BP. However, current literature suggests that placental glucocorticoid signaling could be involved in maternal BP regulation during pregnancy. It was demonstrated in associative clinical studies that HDPs are associated with increased placental glucocorticoid metabolism [7]. Moreover, it was shown that increased maternal glucocorticoid levels induce a reduction in placental expression of vascular endothelial growth factor and its receptors together with impaired angiogenesis [53]. It has been extensively demonstrated that reduced placental perfusion can cause widespread dysfunction of the maternal vascular endothelium, by distinct mechanisms that are not fully understood [54,55]. To better understand the relationship between placental DNA methylation of *NR3C1*, placental glucocorticoid signaling and maternal BP regulation, adequately designed animal experiments, analyzing placentas from different stages of pregnancy, are needed.

A limitation of our study is the usage of placental tissue without focusing on a specific placental cell type. We did not do this, as the purification process on its own – as environmental stimuli – may affect epigenetic alterations of the DNA [56]. Support for a potentially neglectable bias of analyzing whole placenta in DNA methylation studies comes from studies that compared epigenetic profiles of isolated trophoblast cells with whole placental tissue. It was shown by MethylC-seq analysis that global methylation in whole rhesus (Rh) placental tissue was almost identical as compared with isolated Rh trophoblast cell methylation

[57]. Furthermore, Grigoriu *et al.* [58], who investigated cell-specific epigenetic differences between isolated trophoblast cells and fibroblasts using the Illumina450k array, also did not find any differences in regards to cell type specifity of *NR3C1* methylation.

In summary, this study provides evidence of an independent association between *NR3C1* proximal promoter methylation and maternal BP regulation during human pregnancy. Our study provided for the first time evidence that for different patterns of placental *NR3C1* promoter methylation in normotensive, hypertensive and hypotensive pregnancy.

Adequately designed animal studies are necessary to address the question whether the observed alterations are causal in the pathogenesis of BP regulation during pregnancy or a consequence of the altered blood pressure.

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Conflicts of interest

There are no conflicts of interest.

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Reviewer's Summary Evaluation

Reviewer 2

Epigenetic mechanisms are important factors in regulation of gene expression. In complex traits such as hypertension, epigenetic factors can reflect some of the interaction between genes and environment. The present study looked into DNA methylation patterns in normotensive, hypertensive and hypotensive pregnancy focusing on the glucocorticoid receptor gene promoter in human placenta. The authors describe characteristic patterns for these three conditions that do, however, not translate into different gene expression. Despite a number of methodological issues that are appropriately discussed in the paper, the authors provide important descriptive data that pave the way for future mechanistic studies. The paper certainly reminds us yet again of the complexity of glucocorticoid signalling in pregnancy and in general.

DNA_methylation_of_the_glucocorticoid_receptor_gene_promote

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