Very preterm birth is associated with PLAGL1 gene hypomethylation at birth and discharge

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ABSTRACT

Background. Recent findings show that DNA methylation is susceptible to very preterm (VPT) birth and to the experience of the early stay in the Neonatal Intensive Care Unit (NICU). The aim of the study was to compare *PLAGL1* methylation between VPT and full-term (FT) infants at birth as well as between VPT infants at discharge and FT infants at birth.

Methods. DNA was collected from cord blood of 56 VPT and 27 FT infants at birth and from peripheral blood in VPT infants at NICU discharge. Socio-demographic and neonatal variables were considered.

Results. *PLAGL1* methylation at birth and at discharge were highly correlated in VPT infants. Lower methylation emerged in VPT infants at birth and discharge compared to FT counterparts.

Conclusions. *PLAGL1* hypo-methylation emerged as a potential epigenetic mark of VPT birth. Future research is warranted to assess the functional consequences of *PLAGL1* diminished methylation in VPT infants' development.

INTRODUCTION

Very preterm (VPT) birth (i.e. gestational age < 32 weeks) is associated with many negative medical and psychological outcomes both in childhood [1,2] and in adulthood [3]. Notably, even in the absence of perinatal injuries and neurodevelopmental deficits, VPT infants show different developmental trajectories and disrupted long term outcomes in behavioral, physiological and neurological domains [4,5].

Despite there being consistent agreement about the fact that VPT birth represents a risk factor for further adverse developmental trajectories, the mechanisms associated with detrimental outcomes are still under investigation. Researchers to date have investigated environmental [6], physiological [7–9] and genetic factors [10,11] associated with altered developmental outcomes of VPT birth. Specifically, during the last decade, DNA methylation has emerged as a reliable mechanism linking early life adversity with detrimental developmental outcome in infancy, childhood and adulthood [12–14]. Despite it is a normal regulatory process essential to DNA replication and transcription, DNA methylation occurs when a methyl group is covalently attached to cytosine residues in cytosine/guanine (CpG) dinucleotides resulting in reduced transcriptional activity (i.e., gene silencing) [15]. When methylation occurs in a region involved in gene expression and transcription it is of critical relevance as it might produce effects on the actual protein expression and phenotype [15]. Despite the fact that DNA methylation is partially regulated by genetic polymorphisms, such as the *MTHFR* (rs1801133GA) [16,17], it has been shown that epigenetic mechanisms are highly susceptible to environmental manipulations and birth status. Specifically, alterations of DNA methylation – both in terms of increases and decreases – seem to be involved both in premature birth *per se* [18–20] as well as in the negative effects of early exposure to adverse environments connected to prematurity (e.g. procedural pain during NICU hospitalization) on children's further development [21,22]. For instance, Cruickshank and colleagues [23] analyzed DNA methylation retrospectively in a small sample of 18-year-old preterm birth survivors (N = 12) and they documented persistent methylation differences at ten genomic loci comparing preterm subjects with full-term matched controls (N = 12). Increased

methylation at multiple CpG sites including from fetal leukocyte DNA was found in mostly very preterm infants (gestational age range: 24-34 weeks) compared to full-term infants (gestational age higher than 39 weeks) [24].

Imprinted genes, in particular, are considered critical growth effectors responsible for the regulation of fetal development [25]. Greater methylation of these genes has been associated with fetal growth restriction [26] and reduced DNA methylation at the regulatory region of specific imprinted genes (e.g., IGF2, PLAGL1) associates with lower birth weight [27–30]. Prematurity has been retrospectively associated with differences in methylation of imprinted genes in adults born preterm [31]. Prematurity has been retrospectively associated with differences in methylation of imprinted genes in adults born preterm [34]. Moreover, a pool of imprinted genes – including IGF2, MEG3, PEG3, and PLAGL1 – have been found to be differently methylated in infants with low birth weight (< 2500 grams) compared with normal birth weight infants: lower IGF2 methylation was found in infants with low birth weight compared to the normal weight counterpart [28]. Nonetheless, in this prospective study the sample size wid varied among different birth-weight groups of infants (e.g., low birth weight infants, N = 33; normal birth weight infants, N = 475). Other studies investigated DNA methylation at imprinted regions in infants born at low birth weight considering the potential role of maternal (e.g., depression, physical activity) or biological (e.g., infections) factors [28,32,33], but they did not focus specifically on the association between preterm birth and differences in DNA methylation between VPT and full-term infants.

Among imprinted genes, the pleomorphic adenoma gene-like 1 (*PLAGL1*) appears to be of specific interest with regard to prematurity. *PLAGL1* is an imprinted gene that functions as a suppressor of cell growth [34]. Despite the fact that down regulation of this gene in humans is associated with heightened risk of disease, its overexpression is considered a causal factor for transient neonatal diabetes mellitus (TNDM) [35]. Notably, Plagl1 deficient mice present with embryonic growth restriction [36]. Moreover, PLAGL1 gene down-regulation was found in the placenta from intrauterine growth-restriction female, but not male newborns [37]. A recent study [32] focused on the methylation differences of a set of imprinted regulatory

elements in association with preterm birth status (i.e. spontaneous preterm labor, preterm premature rupture of membranes, or medically indicated) and placental infections (chorioamnionitis or funisitis). Results from Liu and colleagues showed significant associations with *PLAGL1* methylation only. Specifically, methylation was higher in preterm infants whose mothers experienced a placental infection compared to their counterparts whose mothers did not experience infection. Interestingly, in another study Liu and colleagues [28] found that infants with high birth weight (> 4.500 kg) were associated with increased *PLAGL1* methylation, however low birth weight infants (< 2.500) were not associated with decreased methylation. Surprisingly, thus far, no study has focused on *PLAGL1* methylation differences between VPT and full-term (FT) infants. In light of the aforementioned paucity in the literature, we hypothesized that a pattern of *PLAGL1* hypo-methylation might be linked with prematurity.

METHODS

Subjects

Fifty-six VPT infants (29 females) and twenty-seven FT infants (12 females) participated in this prospective longitudinal study investigating genetic and epigenetic vestiges of preterm birth and early life adversities (*insert after blind review*). In the present study, we report data on perinatal and discharge methylation of the PLAGL1 gene in VPT infants. VPT infants (gestational age ≤ 32 weeks; range 26-32 weeks) were enrolled at the NICU, XXXX (*insert after blind review*). FT infants were recruited at the Pediatric Unit, XXXX (*insert after blind review*) and participated in only perinatal methylation of the gene. Exclusion criteria for VPT infants were major brain lesions as documented by cerebral ultra-sound, neuro-sensorial deficits, genetic syndromes, and/or major malformations. All FT infants were healthy and had no neonatal morbidities or prenatal/perinatal-risk factors. For mothers, exclusion criteria were the following: age under 18 years, manifest psychiatric and cognitive pathologies, drug addiction, single-parent families. All mothers signed a written informed consent and the study was approved by the Ethics Committees of XXXX (*insert after blind review*) and participating

hospitals. All the procedures followed the guidelines from the Declaration of Helsinki 2013 (World Medical Association).

Protocol

We collected cord blood at birth from full term and preterm infants whereas peripheral blood at discharge was obtained only from preterm infants. Previous research documented that mean methylation values of umbilical cord blood cells resembled those from peripheral blood cells in healthy individuals [38]. Moreover, cord blood sampling has already been used in previous newborn epigenetic studies [11,21,39] and is considered to be non-invasive for human newborns.

Measurements

Infants' perinatal data

Perinatal variables included neonatal (i.e., gestational age, birth weight, gender, Apgar score at minute 1, length of NICU stay) and socio-demographic variables (i.e., maternal age, years of study, occupation status). According to Hollingshead's classification [40], socio-demographic variables were summed up to obtain an index of family socio-economic status (SES). SES score ranged from 0 (occupations that do not require high school graduation) to 90 (occupations that require highly specialized education and training).

PLAGL1 Methylation assessment

Genomic DNA was extracted from whole blood with a GenEluta blood genomic kit (Sigma). We analyzed a 416 base-pairs CpG-rich portion of the *PLAGL1* promoter (chr6:144,328,942-144,329,357, Human hg19 Assembly), between -285 and +131 relative to the transcriptional start site, which contains 57 CpG sites and partially overlaps exon 1.

Methylation levels were determined in DNA using bisulfite modification followed by PCR amplification and NGS. Genomic DNA was extracted from 0.2 ml of each sample using the GenElute Blood Genomic DNA kit (Sigma). Bisulfite conversion was performed on 500 ng of genomic DNA using the EZ DNA methylation kit (ZymoResearch, Inc, Irvine, CA, USA). Primers were designed using Bisulfite Primer Seeker (http://www.zymoresearch.com/tools/bisulfite-primer-seeker). The gene-specific forward 5′-

GTAAGAATYGGGTTTGTGGATTTTATATTAG-3′ and reverse 5′-AACAACCRTACTCACAACTCAACAAC-3′ were used. SureSelect amplicon-specific tails 5′ TGTGATCAAGAGACAG 3′ were added to each primer in order to allow synthesis and sequencing of SureSelect libraries of methylated fragments. Initial PCR-amplifications were performed on 20 ng of bisulfite-treated DNA using Taq Gold (Life Technologies, Inc.). Cycling consisted of 5 min pre-activation at 95°C, followed by 35 cycles of 94°C denaturation for 15s, 58°C annealing for 20s, 72°C elongation for 1.5min. All PCR products were checked on 2% agarose gel and treated with Ilustra Exo Pro-STAR (GE Healthcare) to eliminate unincorporated primers. Adaptors were subsequently attached to each sample by 8 cycles of amplification with a SureSelect Target Enrichment System (Agilent). Samples were again treated with Ilustra Exo Pro-STAR, then indexed using specific forward (i5) and reverse (i7) index primer combinations and the SureSelect protocol. Approximately equimolar aliquots of each product were pooled and purified with AMPure XP beads (Bechman Coulter) following Agilent's protocol. The purified pooled library was quantified on a Bioanalyzer 2100 (Agilent) and sequenced on a MiSeq (Illumina) using a v2 Reagent kit, 300 cycles PE.

In order to analyze the reads, we created a reference sequence of the plus strand of the *PLAGL1* gene mimicking the results of bisulfite modification. The sequence was indexed with the BWA-MEM algorithm. The sequence dictionary was created with the Picard tool (http://broadinstitute.github.io/picard/). We aligned the FASTQ sequence reads, cloned sequences and assembled contigs with the BWA-MEM algorithm. The resulting SAM files were then filtered selecting only reads with a specific cigar value, allowing us to keep only reads which align correctly to the region of interest. We converted the SAM files to BAM files with SAMtools, indexed and sorted them. The reads were visualized with Integrative Genome Viewer (IGV, http://www.broadinstitute.org/igv). Using the IGVtools -count algorithm, we obtained the coverage of each base position in the sequenced region. This allowed us to calculate the percentage of methylated bases presents at each CpG position. The forward read of each sequence covered CpGs 1-17 while the reverse read covered CpGs 38-57. Cpg sites 18-37 were omitted from the analysis due to incomplete or null coverage. CpG site 44

was also omitted because of a C>T polymorphism (rs67300369) in some subjects. The average read depth was 17103. It ranged from 15400 to 18497 between CpG sites. As expected for an imprinted region, CpGs in any single read were coherent, either all methylated or non-methylated. We have verified the coherence for a number of samples by calculating the percentage of methylated CpG positions in each read. Completely coherent imprinted samples would have only 100% (or 0%) methylated reads. The average methylation in the CpG residues of the un-methylated allele reads was 95.6% while methylation in the un-methylated allele reads was 1.5%.

MTHFR polymorphism

As reported by previous studies, a polymorphic genetic variation of the *MTHFR* C677T (rs1801133) might account for individual variability in the degree of DNA methylation (19,20), thus, participant infants were genotyped for this polymorphism. Briefly, 20 ng of genomic DNA from each subject was amplified with primers *MTHFR*-C677T-F (5-CCTCACCTGGATGGGAAAGA-3) and R (5- GCCCCCAAAGCAGAGGACT-3). The resulting 304 bp fragment was sequenced from both ends by Sanger sequencing on an ABI3500AV (Applied Biosystems). The genotype was dummy coded as follows: 0 = GA and AA, 1 = GG. Three subjects (1 VPT and 2 FT infants) had missing information on *MTHFR* genotype. *MTHFR* distribution was 31 GA/AA and 24 GG in the VPT group and 13 GA/AA and 12 GG in the FT group.

Data analysis

Neonatal and socio-demographic characteristics were compared between VPT and FT groups (0 = VPT; 1 = FT) by means of Wilcoxon independent-sample tests and χ^2. Gender (0 = males, 1 = females) related differences in the two groups were tested by means of independent-sample *t*-test. Prior to the analyses, CpG specific methylation values were winsorized to adjust for extreme values and outliers. For the VPT infants' group, CpG-specific methylation at each time point (i.e., birth and NICU discharge) was tested for bivariate correlation. The association between mean methylation and neonatal variables (i.e. gestational age, length of NICU stay) was assessed in VPT infants. We computed different

analyses to compare VPT and FT infants' *PLAGL1* CpG-specific and mean methylation. For what pertains to CpG-specific *PLAGL1* methylation, we reported confidence intervals of the *t*-test for each site through a forest plot. As for *PLAGL1* mean methylation, two regression models were performed. Model A evaluated the effect of birth status (i.e., VPT *vs.* FT) on *PLAGL1* mean methylation at birth, while Model B compared *PLAGL1* mean methylation at comparable term-equivalent age in both groups of infants. Model B included VPT infants' *PLAGL1* methylation at NICU discharge and FT infants' *PLAGL1* methylation at birth. In both models, potential confounders (i.e., infants' gender, maternal age, family SES, *MTHFR* polymorphism and, only for model B, post-conceptional age at the term-equivalent epigenetic sampling). Confounders were selected based on clinical relevance and preliminary account of multicollinearity (measured according to the Variance Inflation Factor, VIF). All analyses were conductedd using R Software [41] setting $p < .05$.

RESULTS

Socio-demographic variables for each of the two subgroups of participants are presented in Table 1. VPTs differed from FTs only in the variables associated with birth status. Gender distribution did not differ between groups ($X^2 = 0.39$, $p = .53$). We have preliminary tested for gender-related differences in PLAGL1 methylation separately in the two groups. In VPT infants at birth, *t*-test was -.87 (p = .39) and it ranged between -1.04 and -.36 (*p*s > .10) for CpG-specific methylation. In VPT infants at discharge, *t*-test was .92 (*p* = .36) and it ranged between .57 and 1.24 (*p*s > .10) for CpG-specific methylation. In FT infants, the t-test for the overall PLAGL1 methylation was -.11 (*p* = .91) and t-test for CpG-specific methylation ranged between -1.03 and .49 (all *p*s > .10).

Insert Table 1 here

High birth-to-discharge correlations emerged for CpG-specific (Figure 1) and mean *PLAGL1* methylation ($r = .61$, $p < .001$) among VPT infants. VPT infants' *PLAGL1* mean methylation at birth and discharge did not correlate with gestational age at birth and length of NICU stay (|*r*s| < .17, *p*s > .21).

Insert Figure 1 here

CpG-specific (Figure 2) and mean *PLAGL1* methylation (Figure 3) significantly differ between VPT and FT infants at birth.

Insert Figure 2 and 3 here

Mean *PLAGL1* methylation was higher in FT infants at birth compared to VPT counterparts at birth (Table 2A) and at discharge (Table 2B). In both regression models (all VIFs < 2.6), no significant effect of the *MTHFR* polymorphism emerged on *PLAGL1* mean methylation.

Insert Table 2 here

DISCUSSION

The present study aimed to assess *PLAGL1* methylation differences between VPT and FT infants. Results suggest that *PLAGL1* methylation is lower in VPT infants compared to their FT counterparts, even when controlling for perinatal confounders. PLAGL1 methylation is known to play a role in cell growth suppression [34]. Although we cannot discriminate between causes and consequences in the present study, our findings might be suggestive of a potential association of PLAGL1 down-regulation and VPT birth which needs to be further tested in future prospective studies including both prenatal and post-natal period assessments. In absence of previous literature linking PLAGL1 methylation and preterm birth the present hypothesis needs to be tested in further studies. Nonetheless, the present findings further contribute to our knowledge of the impact of birth status on the imprinting epigenome of preterm infants, and suggests that altered *PLAGL1* methylation might be considered as a factor involved in preterm delivery [32]. Additionally, meta-analytic evidence suggests that PLAGL1 is a member of a sort of imprinted gene network which comprises multiple genes and other imprinted transcripts that are involved in fetal growth [34,42,43]. As in the present work we only examined methylation of a PLAGL1 region, we cannot directly contribute to the literature on the imprinted gene network [44]. Nonetheless, the notion that the PLAGL1 gene might be involved also in humans in the broader regulation of prenatal and post-natal growth appears consistent with the present findings.

Moreover, *PLAGL1* methylation was found to be lower in VPT infants both at birth and at discharge when their adjusted age would be comparable to a FT infant. In other words, VPT

infants had lower *PLAGL1* methylation when compared to FT infants at birth as well as when accounting for comparable post-conception age. Despite being preliminary, these findings sustain the hypothesis that DNA methylation at imprinting regions – i.e., the *PLAGL1* gene - may potentially provide a deeper understanding of how epigenetic mechanisms contribute to VPT infants' development. Previous research on stress-related genes (e.g., the serotonin transporter gene, *SLC6A4*) showed that the painful nature of NICU-related life-saving procedures (i.e., skin-breaking procedures) is significantly associated with a birth-to-discharge methylation increase (11, e.g. *SLC6A4*,21,22). Conversely, based on the present study, it might be plausible to speculate that imprinted genes, like *PLAGL1*, might be less susceptible to epigenetic regulation as a result of environmental exposures. The persistence of a reduced methylation profile of the *PLAGL1* imprinted ge even at the end of the hospitaliza indirectly supports the hypothesis that the *PLAGL1* hypo-methylation pattern might be inherent to VPT birth status and neither transient nor affected by the length of NICU stay.

Of note, the *PLAGL1* methylation difference observed between VPT and FT infants was not affected by genetic variants of the *MTHFR*. This finding is partially surprising given that, previous research associated the *MTHFR* polymorphism with human variations in methylation [16]. Nonetheless, the *MTHFR* polymorphism was not associated with *PLAGL1* methylation levels, not even in interaction with prematurity. The nature of the interactions between *MTHFR* and imprinted genes deserves further investigation specifically within the context of preterm birth.

The study has limitations. The sample size is relatively small, even if the high homogeneity within the VPT group partially compensates for this limitation. Moreover, only VPT infants were included. Although this choice was made to ensure internal consistency of the findings, results cannot be extended directly to extreme preterm infants (gestational age < 28 weeks) or moderate preterm ones (gestational age > 32 weeks). Finally, due to the observational nature of the study we are not able to distinguish the direction of the association between *PLAGL1* methylation and premature birth (i.e. to determine if *PLAGL1* methylation can be

considered a cause, a consequence or a correlated event of premature birth). Future research should focus on the investigation of the role played by *PLAGL1* methylation in preterm birth and the associated physiologic processes.

In conclusion, this study demonstrates that *PLAGL1* methylation is relatively constant during infants' hospitalization after premature birth. Future studies are warranted to investigate: a) whether *PLAGL1* hypo-methylation is a stable and reliable index of epigenetic processes occurring at imprinting regulatory regions in preterm birth; b) whether altered methylation of imprinted genes is stable across development in VPT infants. Moreover, the functional consequences of reduced *PLAGL1* methylation in VPT infants are not known and the effects of this hypo-methylation profile on health and disease of VPT infants later in life may be a promising line of research for both clinical and research purposes.

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DISCLOSURE

Authors have no conflict of interest.

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FIGURE CAPTIONS

Figure 1. Heat map of the correlations of methylation of each CpG site between birth and discharge for VPT.

Figure 2. Forest plot to show descriptive values of the methylation (proportion) of each CpG site and confrontation between VPT and FT. Note. Blue lines and squares show the comparison between FT methylation at birth and VPT methylation at birth. Red lines and circles show the confrontation between FT methylation at birth and VPT methylation at discharge. FT, full-term; VPT, very preterm.

Figure 3. Differences in mean methylation between groups. Note. FT, Full-Term; VPT, Very Preterm.

SUMMARY POINTS

1. The study of specific genes methylation to research on very preterm (VPT) birth holds promises of being beneficial for scientific knowledge and clinical practice
2. Previous research documented that early stress exposure during the hospitalization might affect the methylation of stress-related genes in preterm infants
3. Here we assessed methylation of an imprinted gene (i.e., *PLAGL1*) involved in fetal development at birth and at discharge in VPT infants and in full-term (FT) controls
4. A significant pattern of hypo-methylation emerged in VPT infants at birth
5. Additionally, this pattern was maintained at discharge, even controlling for variables related to the hospitalization
6. These findings suggest that the methylation of the *PLAGL1* gene might be involved in preterm birth and it might be a stable marker of VPT delivery even at discharge
7. Future basic and clinical studies are needed to assess the behavioral and neurological consequences of *PLAGL1* hypo-methylation in VPT infants

Table 1. Neonatal and socio-demographic characteristics of the sample.

	FT infants N = 27 12 Females		VPT infants N = 56 29 Females		Wilcoxon test	*p*
	Mean	SD	Mean	SD		
Neonatal characteristics						
Gestational age (weeks)	39.62	1.15	30.39	1.88	.50	< .001
Apgar score at minute 1	9.92	.28	6.60	1.49	28.50	< .001
Birth weight (grams)	3402.59	418.91	1395.75	342.21	.00	< .001
Length of NICU stay (days)	---	---	46.23	19.7	---	---
Socio-demographic variables						
Maternal age (years)	33.83	4.41	35.73	3.59	908.00	.14
Maternal education (years of study)	14.81	3.75	16.06	2.33	571.00	.13
Family SES	66.48	20.65	62.14	14.98	635.00	.23
	N	%	N	%		
Causes of preterm birth						
Spontaneous preterm birth	14	25.0	NA	NA		
Spontaneous labor with intact membranes	8	14.3	NA	NA		
Premature rupture of membranes (pPROM)	6	10.7	NA	NA		
Medically indicated preterm birth (maternal/fetal indication)	42	75.0	NA	NA		
Pre-eclampsia	14	25.0	NA	NA		
Intrahepatic cholestasis during pregnancy (ICP)	6	10.7	NA	NA		
Maternal disease [a]	4	7.1	NA	NA		
Placental causes [b]	6	10.7	NA	NA		
Intrauterine growth restriction (IUGR)	5	8.9	NA	NA		

Abnormal blood flow patterns in fetal circulation (fetal distress)	4	7.1	NA	NA
Multiple gestation complicated by twin-twin transfusion syndrome (TTTS)	3	5.4	NA	NA

Note. FT, Full-Term; VPT, Very Preterm; CI, Confidence Interval, NICU, Neonatal Intensive Care Unit; SES, Socio-Economic Status; [a] Acute renal disease or bowel perforation; [b] Placental abruption or placenta praevia.

Table 2. Regression models testing the effect of Birth status (i.e., FT *vs.* VPT) on *PLAGL1* mean methylation at birth (A) and at term-equivalent age (B) controlling for the MTHFR polymorphism.

(A)	R^2	*F*	*p*	*b*	SE	*t*	*p*
Model	.27	5.51	< .001				
Birth status				.09	.02	4.85	< .001
Infant's gender				.01	.02	.66	.51
Maternal age				.001	.002	.55	.59
Family SES				.0005	.0005	.99	.33
MTHFR polymorphism				.01	.02	.32	.75
(B)	R^2	*F*	*p*	*b*	SE	*t*	*p*
Model	.18	2.7	< .05				
Birth Status				.14	.04	3.27	< .011
Post-conception age				.001	.001	1.38	.17
Infant's gender				.03	.02	1.38	.17
Maternal age				.003	.003	.87	.39
Family SES				.0001	.001	.18	.86
MTHFR polymorphism				-.01	.03	-.51	.61

Note. VPT, very preterm; FT, full-term; SES, Socio-Economic Status; Birth status, 0 = VPT, 1 = FT; Infant's gender, 0 = males, 1 = females; MTHFR polymorphism, 0 = GA/AA, 1 = GG.

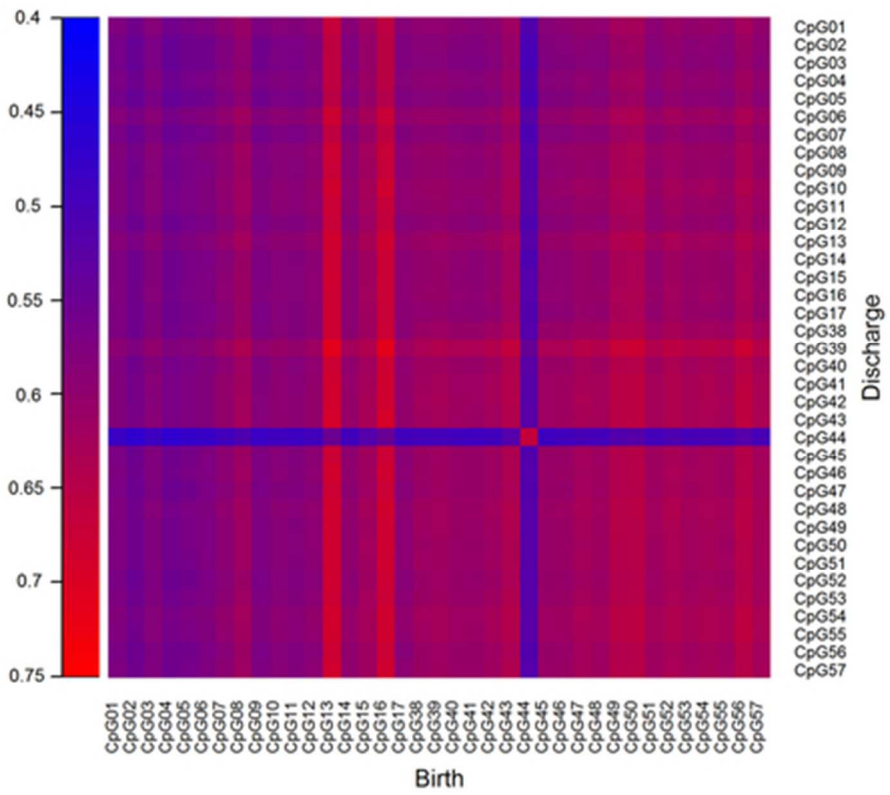

Figure 1. Heat map of the correlations of methylation of each CpG site between birth and discharge for VPT.

37x33mm (300 x 300 DPI)

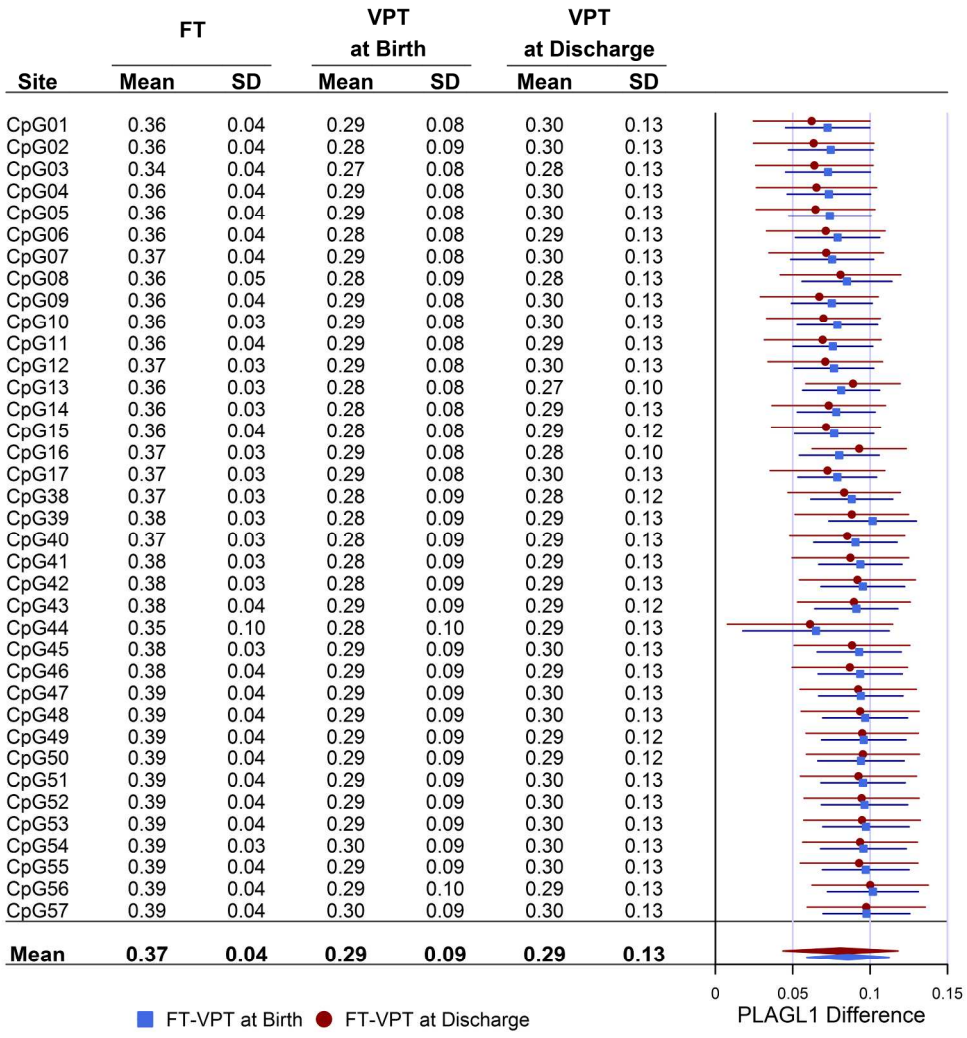

Figure 2. Forest plot to show descriptive values of the methylation (proportion) of each CpG site and confrontation between VPT and FT. Note. Blue lines and squares show the comparison between FT methylation at birth and VPT methylation at birth. Red lines and circles show the confrontation between FT methylation at birth and VPT methylation at discharge. FT, full-term; VPT, very preterm.

228x242mm (300 x 300 DPI)

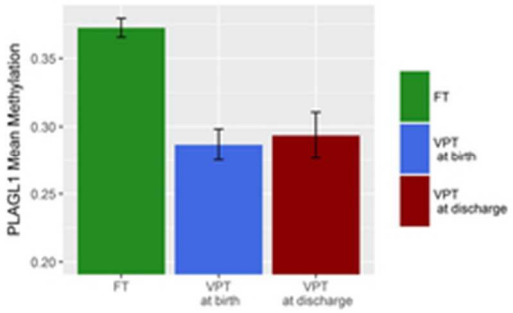

Figure 3. Differences in mean methylation between groups. Note. FT, Full-Term; VPT, Very Preterm.

22x13mm (300 x 300 DPI)