Prenatal vitamin D3 supplementation: pharmacology and offspring health outcomes

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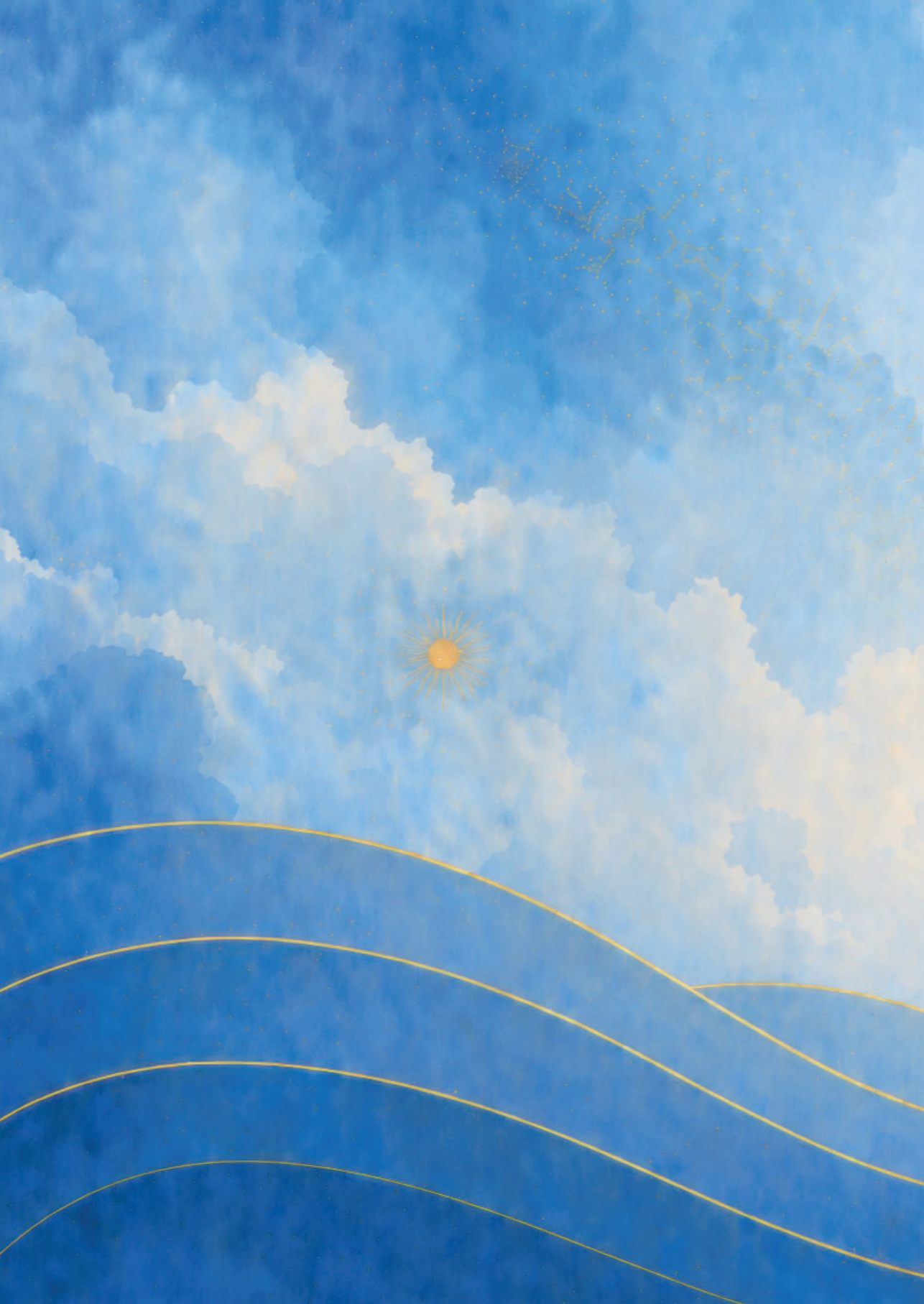

Chapter 8

Cord blood DNA methylation signatures associated with preeclampsia are enriched for cardiovascular pathways: Insights from the VDAART trial

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EBioMedicine. 2023 Dec:98:104890
doi: 10.1016/j.ebiom.2023

Abstract

Background: Preeclampsia has been associated with maternal epigenetic changes, in particular DNA methylation changes in the placenta. It has been suggested that preeclampsia could also cause DNA methylation changes in the neonate. We examined DNA methylation in relation to gene expression in the cord blood of offspring born to mothers with preeclampsia.

Methods: This study included 128 mother-child pairs who participated in the Vitamin D Antenatal Asthma Reduction Trial (VDAART), where assessment of preeclampsia served as secondary outcome. We performed an epigenome-wide association study of preeclampsia and cord blood DNA methylation (Illumina 450K chip). We then examined gene expression of the same subjects for validation and replicated the gene signatures in independent DNA methylation datasets. Lastly, we applied functional enrichment and network analyses to identify biological pathways that could potentially be involved in preeclampsia.

Findings: In the cord blood samples (n=128), 263 CpGs were differentially methylated (FDR<0.10) in preeclampsia (n=16), of which 217 were annotated. Top pathways in the functional enrichment analysis included apelin signaling pathway and other endothelial and cardiovascular pathways. Of the 217 genes, 13 showed differential expression (p's<0.001) in preeclampsia and 11 had been previously related to preeclampsia (p's<0.0001). These genes were linked to apelin, cGMP and Notch signaling pathways, all having a role in angiogenic process and cardiovascular function.

Interpretation: Preeclampsia is related to differential cord blood DNA methylation signatures of cardiovascular pathways, including the apelin signaling pathway. The association of these cord blood DNA methylation signatures with offspring's long-term morbidities due to preeclampsia should be further investigated.

Research in context

Evidence before this study

Preeclampsia can have severe long-term health implication for both mothers and their offspring. However, the pathobiology of these long-term effects remains poorly understood. Maternal DNA methylation alterations in the placenta have been observed in pregnancies with preeclampsia and cord blood DNA methylation changes have been suggested in neonates. These epigenetic changes could be foundational to the long-term offspring morbidity associated with preeclampsia. The effect of the observed methylation marks on neonatal gene expression is currently unknown, but of great interest, as it could shed light on the biological systems involved in preeclampsia's complex pathology.

Added value of this study

We investigated whole genome cord blood DNA methylation of pregnancies complicated with preeclampsia and those without and validated the differentially methylated genes based on their differential expression. We replicated our findings using several preeclampsia cohorts and applied functional enrichment analysis to illuminate biological pathways of preeclampsia-associated genes. This discovery-replication-validation approach identified genes with a high degree of reliability and generalizability. All identified genes were linked to important cardiovascular pathways, representing likely candidates in hypertensive pregnancy disorders such as preeclampsia.

Implications of all the available evidence

Our findings show that genes associated with cardiovascular pathways are differently methylated and expressed in cord blood as a result of maternal preeclampsia. Therefore, these genes serve as potential targets for further investigation of the relation between epigenetic alterations and offspring's cardiovascular health. If a relationship can be established and validated, not only does this provide essential information about the pathophysiology of preeclampsia and its biomarkers, but it also opens the door for therapeutic interventions.

Introduction

Preeclampsia is a complex pregnancy disorder with a variable clinical presentation mainly characterized by hypertension and proteinuria. It is a leading cause of maternal and infant mortality and morbidity, affecting up to 7% of pregnancies.[1] In the United States (US) alone, the short-term health care costs associated with preeclampsia are estimated to be over 6 billion annually.[2] In addition to the short-term health effects, substantial evidence suggests that women with hypertensive disorders of pregnancy (HDP) and their offspring are at increased risk of hypertension and cardiovascular complications later in life.[3-5] Of note, cardiovascular disease (CVD) is the leading cause of death for men, women, and people of most racial and ethnic groups in the US as well as globally, representing 32% of all global deaths.[6,7]

The key pathophysiological abnormalities in preeclampsia are characterized by endothelial dysfunction, metabolic abnormalities, increased oxidative stress, exaggerated inflammatory response, and hypercoagulability.[1] The exact molecular and cellular mechanisms of the disease are not fully understood. However, epigenetic mechanisms, such as DNA methylation, have been implicated in the pathophysiology of preeclampsia.[8] These modifications can be inherited by offspring or acquired in response to changes in the intrauterine environment[9] and could thus potentially mediate the long-term effects of preeclampsia on offspring health.

A recent meta-analysis found that HDP and preeclampsia were associated with multiple DNA methylation marks in cord blood,[10] reinforcing the hypothesis that at least part of the effect of preeclampsia on the offspring might be mediated by alterations in offspring DNA methylation. However, the study did not investigate whether the methylation marks altered gene expression. The study also did not find enrichment of cardiovascular pathways that could be related to the long-term morbidity among the offspring of mothers with preeclampsia.

The contribution of epigenetic regulation in association with the development of CVD has been shown and such an investigation could be more valuable if integrated with transcriptomics.[11] We hypothesized that preeclampsia may influence cord blood DNA methylation patterns in genes that are important for the cardiovascular system, angiogenesis, and endothelial function. Therefore, we examined the association between preeclampsia and cord blood epigenome-wide DNA methylation in a subset of subjects from a well-characterized clinical trial population, the Vitamin D Antenatal Asthma Reduction Trial (VDAART).[12] We then examined whether the associated methylated signatures were also

differentially expressed in the cord blood of the same study subjects in the VDAART cohort. We also used independent datasets of HDP, including preeclampsia, to explore replication of our findings. Lastly, we applied functional enrichment and network analyses to identify potential pathways that could potentially mediate the long-term effects of preeclampsia.

Methods

Study participants

The 128 subjects included in this study were the participants in VDAART who provided samples and agreed to participate in the genomic analyses. Detailed information about the VDAART (clinicaltrials.gov identifier: NCT00920621) design and protocol have been previously published.[12] In brief, the VDAART is a double-blinded multi-center clinical trial that randomized women with singleton pregnancies at 10–18 gestational weeks to receive either high-dose vitamin D_3 supplementation (4,000 IU/day) or placebo in addition to regular prenatal vitamin D_3 supplementation (400 IU/day) to prevent offspring asthma and predefined pregnancy outcomes, including preeclampsia. Subjects in the intervention arms, both the treatment and placebo groups, were included in the present post-hoc analysis. VDAART was approved by the IRBs of the participating institutions (Boston Medical Center, MA; Brigham and Women's Hospital, Boston, MA; Kaiser Health Care San Diego, CA; and Washington University in St. Louis, MO) and a written consent was obtained from all participating women.

Phenotype determination and validation: preeclampsia

Pregnant women with known chronic hypertension were excluded from the trial. Preeclampsia was a prespecified secondary outcome of the VDAART and an adverse event.[13] The study coordinators performed a monthly medical record review for cases of preeclampsia as part of the protocol for identifying serious adverse events in the trial. After delivery, medical records were abstracted for all participants and a committee of four board-certified obstetricians conducted a blinded review of subjects with a noted diagnosis of hypertension, proteinuria, or preeclampsia to determine preeclampsia status. The diagnosis of preeclampsia was based on the American Congress of Obstetricians and Gynecologists (ACOG) guidelines[14] and included the identification of high blood pressure (systolic ≥140 mmHg and/or diastolic ≥90 mmHg at two separate measurements ≥4 hours apart) and either proteinuria (≥300 mg per 24-hour collection or ≥1+ on a urine dipstick) or the presence of elevated liver enzymes, high platelet count, headache, or visual disturbances after 20 weeks of gestation.

Data processing

DNA methylation data processing

Cord blood samples were drawn following the VDAART protocol upon delivery and subsequently persevered at -80°C. DNA was extracted from the cord blood samples of 187 subjects, and bisulfite conversion was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA). We randomized samples by chips and plates and performed genome-wide methylation assays of the bisulfite-treated DNA samples using the Infinium HumanMethylation450 BeadChip array (Illumina, San Diego, CA).[15] A total of 485,512 genome-wide cytosine-phosphate-guanine (CpG) sites were interrogated for each sample and data pre-processing was performed using R v4.1.1[16] and "minfi package" v1.38[17] from Bioconductor. The sample quality control steps included removing samples that were technical replicates, samples with low quality, and samples with genotype or sex mismatches. This led to the removal of 59 out of 187 samples, resulting in 128 included subjects (**Figure 8.1**). The probe quality control steps included removing low-quality probes (detection p-values >0.05), probes on sex chromosomes, single nucleotide polymorphism (SNP) probes, probes within 10bp of a known SNP with minor allele frequency ≥1%, non-CpG probes, as well as non-specific and cross-reactive probes. We applied the normal-exponential out-of-band method for background correction and dye bias adjustment ('noob') and normalized the data using functional normalization ('funnorm'). The final number of probes for analysis was 311,566 (64%) for 128 subjects. The methylation values (β value with a range 0–1) were calculated using β = meth / (meth + unmeth + offset) and used in the differential methylation analyses. Each CpG site was annotated with the associated genes based on the data provided by the University of California Santa Cruz (UCSC; Genome Browser assembly ID: hg38) using the package "IlluminaHumanMethylation450k.db" v1.4.6.[18] from Bioconductor.

Epigenome-wide statistical inflation was evaluated by calculating the genomic inflation factor lambda (λ) and the quantile-quantile plot (QQ-plot) was generated. A predefined λ of ≥1.2 was considered as an indication of significant inflation and the QQ-plot was used in addition to visualizing possible deviations from the null expected distribution.

Gene expression data processing

Of the 187 subjects with cord blood methylation data in the VDAART, 170 also had available samples for gene expression profiling using a microarray platform (**Figure 8.1**). In these samples, total RNA was isolated from whole blood using the QIAGEN PAXgene Blood RNA Kit

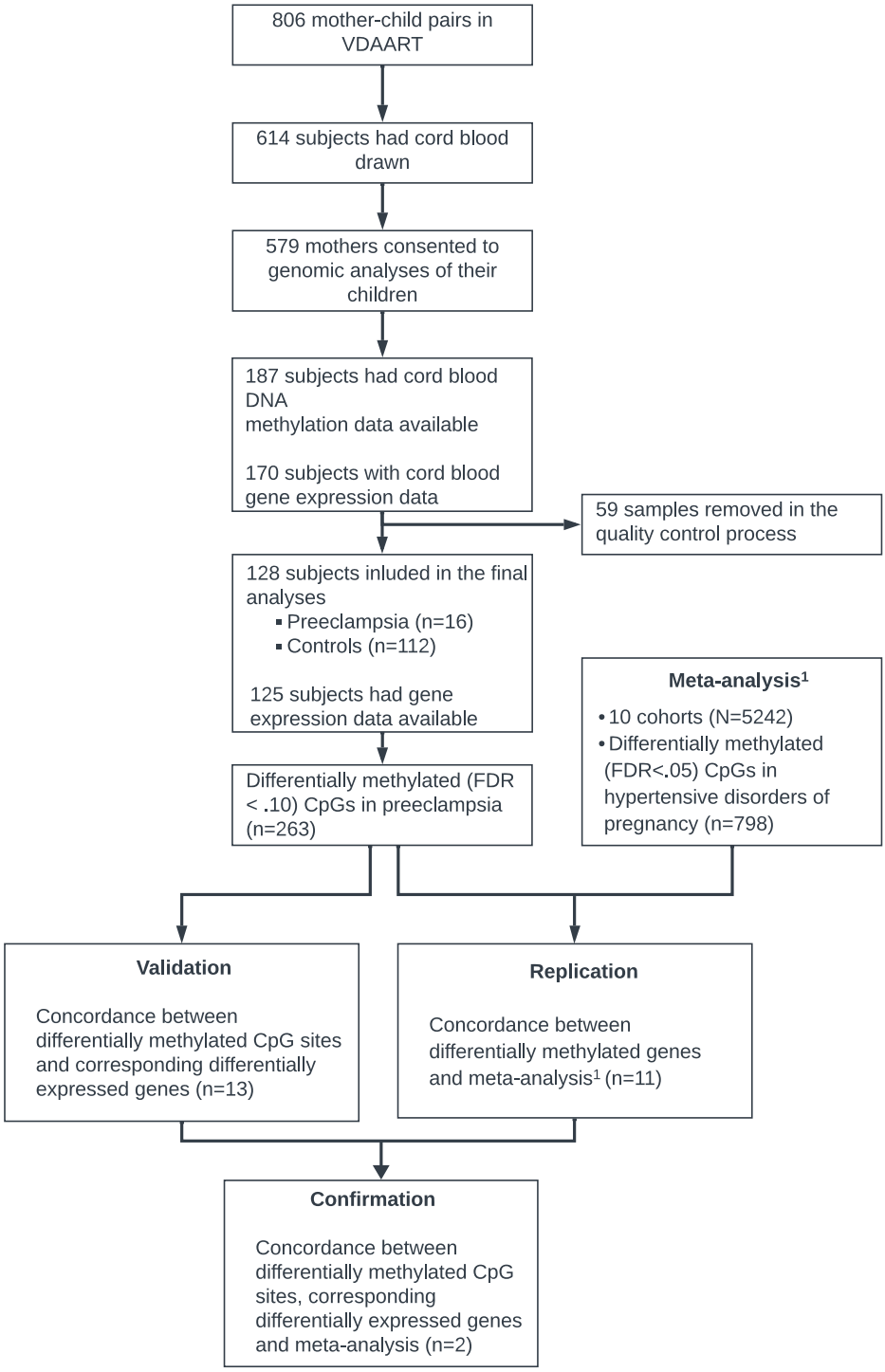

Figure 8.1: Flow chart of the study.

according to the manufacturer's protocol. The GLOBINclear Kit (Ambion) was used to remove α and β globin mRNA from the sample to increase the sensitivity of the gene expression assays by improving the detection rate of expressed genes. The RNA was quantified using the Nanodrop 8000 and checked for high integrity before the preparation of cDNA. The integrity of RNA samples was assessed using the Agilent 2100 Bioanalyzer, and the purity of the samples was confirmed using the NanoDrop spectrophotometer and RNA integrity number (RIN $\geq$8). Gene expression was assessed using the Affymetrix Human Gene 1.0 ST Array. Biotinylated cRNA was prepared according to the manufacturer's protocol, and hybridization was processed according to the protocol for the GeneChip Hybridization Control Kit.

We examined the quantiles of raw expression and principal components across arrays before and after background adjustment normalization and $\log_2$ transformation. The arrays were background adjusted, $\log_2$ transformed, and quantile normalized using the robust multiarray analysis ("rma") function in R from Bioconductor's "affy" v1.75 library.[19] Probes were annotated using the Bioconductor package "hugene10sttranscriptcluster.db" v8.8[20] and the expression set was limited to probes annotated for autosomal chromosomes. Then, the interquartile range (IQR) filter, which included values encompassing 80% of the probe expressions, was applied using the "genefilter" package (v1.79) from Bioconductor.[21] This step was undertaken to increase power by eliminating expressions that exhibited minimal variation within arrays. Surrogate Variable Analysis was applied using the Bioconductor "sva" v3.45[22] package to account for sources of expression heterogeneity and potential latent confounders (i.e. batch effect) in addition to the primary confounders. The principal component analysis was performed, and one outlier sample was detected in the expression level intensity plots using signal-to-noise ratio (95th percentile of signal divided by 5th percentile) across array plots. This sample was removed which resulted in a final expression set of 18,881 gene probes profiled in 169 subjects.

Statistics

DNA methylation data analysis

The epigenome-wide association study (EWAS) of preeclampsia and cord blood DNA methylation was performed using a robust linear model ('rlm' function) developed in R v4.1.1[16] using the package "MASS" v7.3-57.[23] The assumptions of robust linear regression were checked using diagnostic plot functions implemented in MASS. Specifically, these plots ensured linearity, independence of residuals, homoscedasticity, and normality of residuals.

This robust approach is less susceptible to outliers, which are common in DNA methylation data, particularly CpG sites that have low levels of variation or are located at the extreme ends of the distribution. We used methylation (β-values) as the outcome and preeclampsia as the predictor adjusting for the potential confounders. These variables included VDAART intervention arm, maternal race (Black, White, or other), maternal age (≤35 years or >35 years), fetal sex (male or female), gestational age at delivery (<37 gestational weeks or ≥37 gestational weeks), and cell type proxies[24] estimated for cord blood (percentages of monocytes, granulocytes, CD8T cells, CD4T cells, B cells, and nucleated red blood cells). Cell type proxies were only used for methylation analyses. False Discovery Rate (FDR) was calculated using the Benjamini-Hochberg Procedure to select the DNA methylation marks for validation using gene expression analysis. An FDR significance cutoff of 10% was applied to increase the number of potential methylation marks candidates. We based this decision on the availability of resources for rigorous subsequent validation and replication processes, which allowed for careful further validation or exclusion of any initially significant findings. This approach strikes a balance between the sensitivity of the analysis and correction for false positives, aligned with the hypothesis generating nature of the study and enhanced our ability to potentially detect subtle epigenetic changes that could elude detection with a more stringent FDR.

Post-hoc power analysis using the pwrEWAS package (v1.14.0)[25] revealed that with a sample size of 128 subjects, including 16 pregnancies complicated by preeclampsia, there was approximately 80% statistical power at an FDR threshold of less than 0.1. At this level of power, a minimum 2% difference in DNA methylation levels could be detected across 300,000 CpGs, with an emphasis on 300 differentially methylated target CpGs. These calculations considered a 5% standard deviation (SD) in DNA methylation levels within cord blood samples.[26] **Online Figure 1** shows study power estimates across a range of SDs.

Gene expression data analysis

Cord blood gene expression (Affymetrix Human Gene 1.0 ST Array) sample preprocessing steps are described in the supplementary material. Differential expression analysis of the annotated genes corresponding to the methylated signatures identified in the primary EWAS was performed using a robust linear model in the R package "MASS"[23] with expression as the outcome and preeclampsia as the predictor adjusted for maternal race, maternal age, fetal sex, VDAART intervention arm, and gestational age at delivery. A p-value threshold of <5% was used for this validation analysis.

Replication of DNA methylation signatures

We used a meta-analysis conducted by Kazmi and colleagues,[10] which examined cord blood DNA methylation differences in HDP based on data from 10 independent cohorts with a total of 5,242 newborns (preeclampsia: 3 cohorts; n=2,219 [cases=135]),[27-34] to investigate the concordance of our findings in VDAART with previously published data. All cohorts included in the meta-analysis utilized the Illumina 450k BeadChip for methylation analysis,[15] which aligns with the methodology used in the present analysis. The meta-analysis reported a total of 1,075 differentially methylated CpGs (255 associated with preeclampsia) that met the 5% FDR. Of these sites, 798 were annotated and used as the replication dataset.

Functional enrichment and biological pathway analysis of methylation marks

The R package "missMethyl" v1.25.0[35] in Bioconductor was used to test for the enrichment of KEGG pathways in the differentially methylated CpGs identified in our primary EWAS of preeclampsia in VDAART. Appropriately, this statistical package considers the number of CpGs per gene to avoid a potential bias due to the different numbers of CpG sites profiled for each gene. Pathways with ≥2 differentially methylated CpGs and a confidence level of >95% were regarded as significantly enriched. The R package "STRINGdb" v2.11.5[36] with Gene Ontology (GO) and KEGG pathway annotations was used for gene-based enrichment analyses and enriched pathways were reported at FDR<0.05.[36] This package was also used for mapping of signature genes onto the protein-protein interaction (PPI) network and network analysis. The STRING database contains predictions of functional PPIs (indirect association) in addition to physical annotations. These predictions are based on several features, including gene co-occurrence in genomes (i.e. phylogenetic tree), gene co-expression, gene fusion events, genomic neighborhood, text mining, and experimental data. All predictions are combined into a single score for each gene-gene interaction, which ranges from 0 to 1. We used a score with at least medium confidence in prediction (>0.4) that integrates the various available evidence on functional interaction between two proteins to map the PE gene signature. Accordingly, 563,274 biological interactions between 19,566 human proteins were included for the construction of PE module from the overlapping gene signatures.

Ethics

All included patients were enrolled in the VDAART study (ClinicalTrials.gov ID NCT00902621), approved by the institutional review board of Brigham and Women's Hospital and at each participating institution. All women provided written informed consent prior to study inclusion. All analyses were performed in accordance with local and international regula-

tions for research ethics in human subject research. This study conformed to the principles of the Helsinki Declaration.

Role of the funding source

This study is a secondary analysis of VDAART project. VDAART received funding from the National Heart, Lung, and Blood Institute (NHLBI) of the National Institutes of Health in the United States (grant references U03 HL091528 and R01 HL091528). The funding source played no role in the study's design, data analysis, interpretation, or the preparation of this manuscript.

Results

Study cohort characteristics

Figure 8.1 illustrates the flow chart of the study and baseline characteristics of the study subjects are shown in **Table 8.1**. Of the 806 mother-child pairs in the VDAART cohort, 128 had available cord blood DNA methylation data that passed all sample preprocessing steps and 125 of these subjects also had available cord blood gene expression data. Of the study subjects, 16 (13%) had preeclampsia of which 1 (6%) fulfilled the criteria of preeclampsia with severe features.[37] The baseline characteristics of subjects with or without preeclampsia did not differ significantly (**Table 8.1**). The characteristics of subjects included and excluded from the ITT VDAART cohort (**Online Table 1**) displayed differences for maternal age, maternal race, and clinical center. To account for these disparities, we included adjustments for maternal age and race in all our analyses. We chose not to include clinical center in our models, due to its notably strong association with race. **Online Table 2** demonstrates the comparison between preeclamptic cases and normal pregnancies from the ITT cohort with the primary outcome of VDAART (N=806).

Cord blood DNA methylation in preeclampsia

The EWAS of preeclampsia and cord blood DNA methylation in the VDAART identified 263 differentially methylated CpGs (FDR<0.1) (**Table 8.2** and **Online Table 1**) of which 217 (82.5%) were gene annotated (**Figure 8.2**). Preeclampsia was associated with hypermethylation in 207 (79%) of the 263 signature marks. Visual inspection of the QQ-plot (**Online Figure 2**) did not show evidence of significant inflation and the λ was at an acceptable level (1.13).

Top pathways in the KEGG enrichment analysis of the 263 differentially methylated CpGs were endocrine resistance (FDR<0.001) and apelin signaling pathways (FDR=0.001) (**Table**

8.3). Other enriched pathways included platelet activation, Th_1 and Th_2 cell differentiation, fluid shear stress and atherosclerosis, breast cancer, arginine biosynthesis, and relaxin signaling (FDR<0.05).

Table 8.1: Baseline characteristics of the study subjects (n=128) stratified by maternal preeclampsia

Characteristic	Preeclampsia (n=16)	No preeclampsia (n=112)	p-value
Maternal age in years, mean (SD)	26.4 (5.9)	25.8 (5.6)	0.704
<30 years, N (%)	12 (75.0)	84 (75.0)	0.582
30–34 years, N (%)	2 (12.5)	21 (18.8)	
≥35 years, N (%)	2 (12.5)	7 (6.2)	
Maternal race, N (%)			0.370
Black	12 (75.0)	66 (58.9)	
White	3 (18.8)	41 (36.6)	
Other	1 (6.2)	5 (4.5)	
Household income, N (%)			0.410
<$50,000	5 (31.2)	52 (46.4)	
≥$50,000	4 (25.0)	28 (25.0)	
Unknown	7 (43.8)	32 (28.6)	
Parity, mean (SD)	2.3 (1.5)	2.3 (1.5)	0.873
Gestational diabetes, N (%)	0	4 (3.6)	1.000
Vitamin D insufficiency at enrollment†	15 (93.8)	88 (78.6)	0.194
Vitamin D treatment (4,400, IU/daily), N (%)	7 (43.8)	59 (52.7)	0.688
Clinical center			0.488
Boston	4 (25.0)	22 (19.6)	
San Diego	1 (6.2)	20 (17.9)	
St. Louis	11 (68.8)	70 (62.5)	
Gestational age at delivery			
Age (weeks, mean (SD))	38.7 (1.8)	39.0 (1.4)	0.446
<37 weeks, N (%)	3 (18.8)	6 (5.4)	0.151
Fetal sex (male, N (%))	11 (68.8)	52 (46.4)	0.161
Preeclampsia with severe features‡	1 (6%)	N.A.	N.A.

Differences between the groups analyzed using Student's t-test, Chi-Squared test, or Fisher Exact test. Data presented as mean (SD).

† Plasma 25-hydroxyvitamin D level of <30 ng/ml at study enrollment (10–18 gestational weeks).

‡ Preeclampsia with severe features was defined based on the American College of Obstetricians and Gynecologists practice guidelines for clinical management of gestational hypertension and preeclampsia.[25]

Validation with cord blood gene expression data in VDAART

Cord blood gene expression levels were available for 200 (92%) of the 217 differentially methylated genes in the VDAART (the 17 genes not found in the expression dataset are provided in **Online Table 2**). Of these 200 genes, 13 were differentially expressed in preeclampsia (p<0.05) (**Table 8.4** and **Online Figure 3**). Effect directions of the methylation and

expression levels were largely opposite, i.e., hypermethylated genes were suppressed and hypomethylated genes were overexpressed in preeclampsia, except for four genes which were hypermethylated and overexpressed in preeclampsia.

Table 8.2. Epigenome-wide association study* of preeclampsia and cord blood DNA methylation in the VDAART (n=128). Top 30 of the 263 differentially methylated CpGs are presented.

CpG	Gene	Chr	Coef	SE	p-value	FDR
cg27461259	*PPARGC1A*	4	0.067	0.011	2.42E-09	0.001
cg02033213	*CNTN1*	12	0.019	0.003	6.12E-09	0.001
cg03064400	*U2AF1L4*	19	0.016	0.003	7.58E-09	0.001
cg09300856	*NAGS*	17	0.020	0.004	1.06E-08	0.001
cg01510903	*KRT18*	12	0.005	0.001	2.73E-08	0.001
cg18073380	*ZBTB11*	3	0.008	0.001	3.27E-08	0.001
cg20922660	*PRR3*	6	0.010	0.002	3.33E-08	0.001
cg09008705	*NGF*	1	0.013	0.002	5.68E-08	0.002
cg06742228	*MDH1B*	2	0.012	0.002	5.83E-08	0.002
cg20715326	*HYOU1*	11	0.012	0.002	1.01E-07	0.003
cg01653672	*RBCK1*	20	0.013	0.003	1.19E-07	0.003
cg05601456	*ARSJ*	4	0.019	0.004	1.41E-07	0.004
cg26463200	*PAX1*	20	0.011	0.002	1.54E-07	0.004
cg01395760	*KIF22*	16	0.019	0.004	1.90E-07	0.004
cg15360853		17	0.007	0.001	2.06E-07	0.004
cg00918762	*MRPS18B*	6	0.012	0.002	2.59E-07	0.005
cg15054260	*RIPK2*	8	0.011	0.002	2.82E-07	0.005
cg16595223		19	0.006	0.001	4.13E-07	0.007
cg11755107	*C8orf41*	8	0.009	0.002	4.64E-07	0.008
cg12665003	*LPCAT3*	12	0.009	0.002	4.86E-07	0.008
cg18533397		1	0.008	0.002	6.88E-07	0.010
cg20835282	*C3orf62*	3	0.011	0.002	7.25E-07	0.010
cg06285590		11	0.007	0.001	8.06E-07	0.010
cg16163756		2	-0.011	0.002	8.06E-07	0.010
cg10905918	*RPS24*	10	0.010	0.002	9.78E-07	0.012
cg25328923	*LRRC37A3*	17	0.008	0.002	1.17E-06	0.014
cg04983296	*PAQR3*	4	0.014	0.003	1.27E-06	0.015
cg21400015	*ONECUT2*	18	0.010	0.002	1.34E-06	0.015
cg13399952	*FNBP1*	9	0.023	0.005	1.39E-06	0.015
cg20884939		10	0.008	0.002	1.44E-06	0.015

* The differential methylation analysis was performed using a robust linear model with methylation as the outcome and preeclampsia as the predictor adjusted for maternal age at enrollment (>35 vs. ≤35 years), fetal sex, maternal race (Black vs. White vs. other), gestational age at delivery (<37 vs. ≥37 gestational weeks), trial intervention arm (high-dose gestational Vitamin D_3 supplementation vs. placebo), and cell type estimations. Abbreviations: Chr - Chromosome; Coef – Coeffficient; SE – Standard Error; FDR – False Discovery Rate.

Table 8.3: KEGG pathway enrichment analysis of the 263 differentially methylated CpGs in preeclampsia in the VDAART (n=128)

Pathways	CpGs identified	CpGs in pathway	p-value
Endocrine resistance	8	95	<0.001
Apelin signaling pathway	8	135	0.001
Shigellosis	10	233	0.002
Progesterone-mediated oocyte maturation	6	86	0.002
Neurotrophin signaling pathway	7	114	0.003
Platelet activation	7	123	0.003
Prolactin signaling pathway	5	70	0.004
Gastric acid secretion	5	75	0.009
Th_1 and Th_2 cell differentiation	5	86	0.010
Fluid shear stress and atherosclerosis	6	134	0.013
AMPK signaling pathway	6	117	0.013
Breast cancer	7	145	0.014
Longevity regulating pathway	5	88	0.015
Hedgehog signaling pathway	4	55	0.016
Longevity regulating pathway - multiple species	4	61	0.018
TNF signaling pathway	5	108	0.019
Chemical carcinogenesis - receptor activation	7	196	0.019
Arginine biosynthesis	2	19	0.029
Coronavirus disease - COVID-19	6	205	0.029
Cellular senescence	6	152	0.033
Cholinergic synapse	5	111	0.039
NOD-like receptor signaling pathway	5	155	0.039
Growth hormone synthesis (secretion and action)	5	115	0.039
PD-l1 expression and PD-1 checkpoint pathway in cancer	4	88	0.040
Relaxin signaling pathway	5	124	0.042
Oxytocin signaling pathway	6	151	0.043
Cocaine addiction	3	47	0.048
Yersinia infection	5	129	0.049

Analysis was performed using the R package missMethyl to test for KEGG pathway enrichment of the 263 differentially methylated CpGs identified our EWAS of preeclampsia in VDAART. Pathways with ≥2 differentially methylated CpGs and a confidence level of >95% are presented.

Replication of the cord blood DNA methylation in independent cohorts

Of the 217 differentially methylated genes in the VDAART, 11 were associated with HDP in the meta-analysis of independent datasets by Kazmi and colleagues (**Online Table 3**). Cross-validating the 11 genes that were in concordance between our analysis and the external replication cohort, we found that two genes, *FOS* and *WWTR1*, were also differentially expressed in the VDAART cord blood expression set.

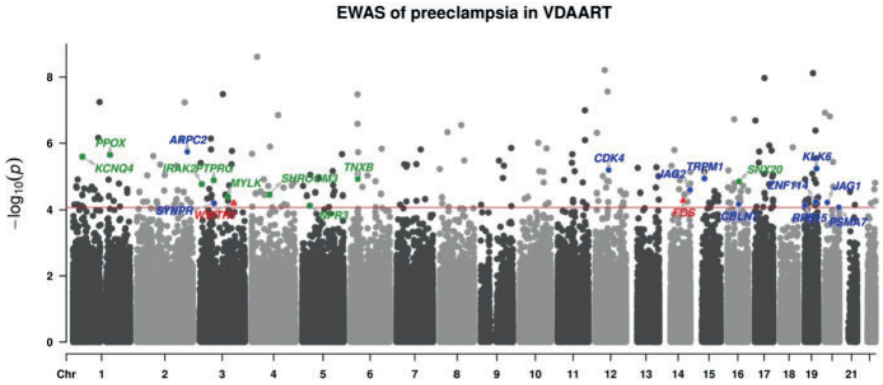

Figure 8.2: Manhattan plot for the epigenome-wide association study of preeclampsia and cord blood DNA methylation in the VDAART (n subjects = 128). The red line indicates an FDR cutoff of 10% above it. Genes highlighted in blue were concordant with the differential expression analysis in VDAART (validation), those highlighted in green were concordant with the meta-analysis by Kazmi and colleagues.[7] (replication), those highlighted in red were concordant with both the validation and replication analyses.

Table 8.4: Overlap between differentially methylated (FDR<0.10, n=263) and differentially expressed (p's<0.05) genes associated with preeclampsia in the VDAART (n subjects = 125)

Gene	Chr	Methylation coefficient	Methylation p-value	Methylation FDR	Expression coefficient	Expression p-value
ARPC2	2	0.008	1.82E-06	0.015	-0.131	1.93E-02
KLK6	19	-0.037	5.85E-06	0.028	0.083	1.50E-02
CDK4	12	0.006	6.51E-06	0.031	-0.160	7.53E-05
TRPM1	15	-0.047	1.17E-05	0.041	0.059	3.99E-02
JAG2	14	-0.009	2.59E-05	0.058	0.090	3.62E-02
FOS	14	0.006	5.22E-05	0.082	-0.316	6.75E-03
ZNF114	19	0.006	5.70E-05	0.084	0.059	2.53E-02
JAG1	20	0.015	5.98E-05	0.085	0.117	2.78E-02
WWTR1	3	0.005	6.25E-05	0.087	0.077	4.87E-02
SYNPR	3	0.045	6.31E-05	0.087	-0.043	3.84E-02
CBLN1	16	0.003	6.66E-05	0.090	0.097	3.83E-02
RPS15	19	0.006	7.26E-05	0.093	-0.111	2.07E-02
PSMA7	20	0.006	8.40E-05	0.099	-0.136	1.46E-03

* The differential methylation/expression analyses were performed using a robust linear model with methylation/expression as the outcome and preeclampsia as the predictor adjusted for maternal age at enrollment (>35 vs. ≤35 years), fetal sex, maternal race (Black vs. White vs. other), gestational age at delivery (<37 vs. ≥37 gestational weeks), trial intervention arm (high-dose gestational Vitamin D_3 supplementation vs. placebo). The differential expression analysis was also adjusted for cell type estimations. Abbreviations: Chr - Chromosome; FDR – False Discovery Rate.

Functional enrichment and network analyses

In total, 22 genes overlapped between the VDAART EWAS and differential expression analysis (validation) or the VDAART EWAS and the HDP methylation meta-analysis (replication). Mapping of these 22 genes onto the human PPI network (interactome) identified

3 clusters of interacting genes, each consisting of 2–4 nodes, and 13 disconnected noted (**Online Figure 4**). Applying the shortest path algorithm,[38,39] we identified 10 closest genes in the neighborhood of 22 gene signatures that connected the 3 clusters. Addition of these neighboring genes to the mapped 22 gene signatures constructed the preeclampsia module in the cord blood with the largest connected component (LCC) of 22 nodes (**Figure 8.3**). This cluster included *WWTR1* and *FOS* as well as other genes related to cardiovascular function including *NOTCH1*, *EGFR*, *EGF*, and *JAG1-2*. KEGG enrichment analysis linked the 22 genes to several pathways identified in the EWAS enrichment analyses including the apelin signaling pathway, endocrine resistance, breast, and other cancer pathways, Th_1 and Th_2 differentiation, and prolactin signaling pathway (Top 30 pathways are presented in **Table 8.5** and all significantly enriched pathways in **Online Table 4**).

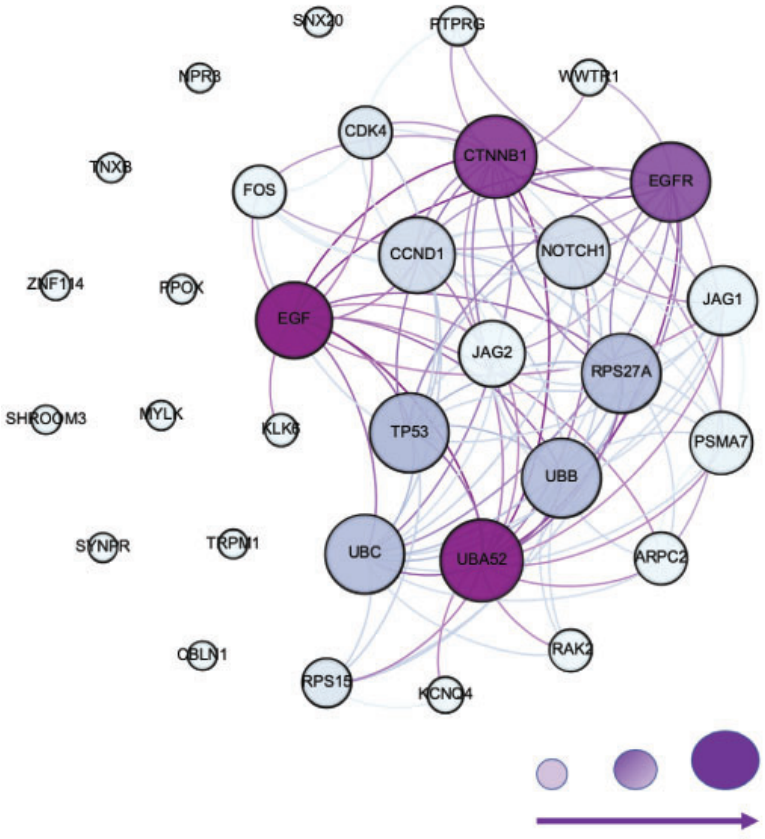

Figure 8.3: Protein-protein-interaction network of the 22 overlapping gene signatures between the differential methylation analysis in the VDAART and the differential expression analysis in VDAART (validation) and/or the meta-analysis by Kazmi and colleagues.[7] (replication). After adding the 10 genes in the vicinity of the 22 overlapping gene signatures in the interactome, the preeclampsia module was constructed from 32 genes with the largest connected component of 22 genes showing direct interactions. The size and darkness of the nodes of correspond to the degree connectivity and betweenness centrality of that node, respectively.

Table 8.5: KEGG pathway enrichment analysis of the 22 genes identified in the preeclampsia EWAS in VDAART and in the validation and/or replication analyses

# Background genes in pathways	# Identified genes	Description	p-value	FDR value
147	10	Breast cancer	4.52E-14	5.38E-12
95	8	Endocrine resistance	4.18E-12	2.49E-10
317	9	Human papillomavirus infection	1.75E-09	6.94E-08
161	7	Apoptosis	8.43E-09	1.01E-07
85	6	Colorectal cancer	6.97E-09	1.97E-07
515	10	Pathways in cancer	6.64E-09	1.97E-07
41	5	Bladder cancer	1.09E-08	2.17E-07
12	4	Ubiquitin	1.05E-08	8.17E-07
10	4	Ubiquitin conserved site	5.79E-09	8.17E-07
58	5	Endometrial cancer	5.50E-08	9.35E-07
66	5	Non-small cell lung cancer	1.01E-07	1.50E-06
68	5	Glioma	1.16E-07	1.54E-06
72	5	Melanoma	1.52E-07	1.81E-06
74	5	Pancreatic cancer	1.73E-07	1.87E-06
27	5	Nucleotide-binding oligomerization domain containing signaling pathway	1.63E-09	3.63E-06
32	5	Regulation of transcription from RNA polymerase II promoter in response to hypoxia	3.51E-09	3.63E-06
67	6	Regulation of transcription from RNA polymerase II promoter in response to stress	1.82E-09	3.63E-06
2,198	17	Cell surface receptor signaling pathway	8.02E-09	3.63E-06
33	5	MyD88-dependent toll-like receptor signaling pathway	4.04E-09	3.63E-06
14	4	Intracellular transport of virus	1.76E-08	4.35E-06
964	12	Regulation of transferase activity	1.95E-08	4.35E-06
183	6	Kaposi's sarcoma-associated herpesvirus infection	5.47E-07	5.42E-06
97	5	Prostate cancer	6.23E-07	5.70E-06
51	5	Interleukin-1-mediated signaling pathway	3.01E-08	6.09E-06
197	6	Focal adhesion	8.30E-07	7.06E-06
19	4	Error-free trans lesion synthesis	5.07E-08	8.66E-06
1,052	12	Response to abiotic stimulus	5.06E-08	8.66E-06
20	4	Error-prone trans lesion synthesis	6.08E-08	9.01E-06
340	8	Positive regulation of protein serine/threonine kinase activity	6.62E-08	9.20E-06
488	9	Regulation of protein serine/threonine kinase activity	6.74E-08	9.20E-06

Abbreviations: FDR – False Discovery Rate.

FOS and *WWTR1* were two genes that were found to be common between all three stages of EWAS, gene expression analysis and replication of annotated CpGs in independent cohorts but did not show direct interaction on the PPI network. Applying the shortest path algorithm, we identified 9 closest neighboring genes that connected these two genes,

constructing a subnetwork of the preeclampsia module with 11 genes (**Online Figure 5**). KEGG enrichment analysis of these 11 genes showed significant enrichment of several biological pathways identified in the main analysis including the apelin signaling pathway, breast cancer, and endocrine resistance (**Online Table 5**). However, the top pathways were related to a transcription factor,

Adenosine 3′,5′-cyclic monophosphate (cAMP) and calcium ion signaling. More notably, several pathways related to cardiovascular function, including circulatory system process, natriuretic peptide receptor activity, blood pressure regulation, and cardiac conduction, were also enriched.

Discussion

In the present study, we found that preeclampsia is associated with differential DNA methylation signatures in cord blood that show significant enrichment to functional and developmental cardiovascular pathways, particularly the apelin signaling pathway. Enrichment of these pathways was also observed when limiting our focus to genes that were validated by examining the cord blood gene expression levels or replicated in independent datasets. These findings warrant further investigation into a potential association between DNA methylation and long-term cardiovascular morbidity in the offspring of mothers with preeclampsia.

Intrauterine exposure to preeclampsia is associated with a wide variety of potentially lasting health effects in the offspring.[40] In particular, it has been shown that offspring born to pregnancies complicated by preeclampsia experience a higher incidence of congenital heart defects[41] and demonstrate risk factors for CVD, such as increased BMI, hypertension, and altered vascular function.[3,42,43] Epigenetic alterations have been proposed as the mechanistic link between preeclampsia and offspring CVD, although the exact nature of this relationship is yet to be elucidated.[44,45]

The apelin signaling pathway was one of our main findings in the CpG-based enrichment analysis of our primary EWAS and significantly enriched in the gene-based analysis of the overlapping genes of our primary EWAS and the validation/replication analyses. The apelin system participates in a diverse array of processes, including glucose metabolism, immune function, and fluid homeostasis.[46] However, its principal physiological role seems to be related to its cardiovascular actions and angiogenesis; in humans, apelin peptides induce peripheral and coronary vasodilatation while increasing cardiac output and contrac-

tility.[47] The apelin signaling pathway has also been implicated in the pathophysiology of preeclampsia[48] and animal models have demonstrated favorable outcomes after apelin administration in preeclamptic pregnancies.[49] Our findings indicate that offspring of mothers with preeclampsia may demonstrate differential methylation of the pathway already at birth.

The AMP-activated protein kinase (AMPK) signaling pathway was also significantly enriched in our differential methylation analysis of preeclampsia. The AMPK pathway regulates the contraction of vascular smooth muscle cells via nitrogen oxide-induced vasorelaxation and plays an important role in regulation of vascular tone and blood pressure control.[50,51] It also has anti-atherosclerotic effects mediated by nitrogen oxide bioavailability, reactive oxygen species stress responses, and proangiogenic factor activation.[50] Interestingly, the cGMP signaling pathway was also significantly enriched in our constructed module in the PPI network from the annotated genes. Cyclic GMP is a critical downstream effector of the inducible nitric oxide pathway and atrial natriuretic peptide with a pivotal role in vascular smooth muscle cell contractility, and thus vascular wall physiology and blood pressure regulation.[52] Furthermore, it directs an anti-proliferative signal in vascular smooth muscle cell proliferation and differentiation. Within the myocardium it inhibits hypertrophy and regulates contractility.[52] One of the differentially methylated genes in our study and in the meta-analysis of Kazmi and colleagues10 was natriuretic peptide receptor 3 (*NPR3*) which encodes the natriuretic peptide clearance receptor. *NPR3* polymorphism has been related to blood pressure control and the risk of hypertension.[53,54] The exact mechanisms of this effect remain unclear but are thought to be related to natriuretic peptide clearance[54] and vascular smooth muscle cell functions.[55,56]

Notch signaling plays an indispensable role in the angiogenic process of placental development through downstream activation of the transcription of a set of genes, but most notably Hairy/enhancer-of-split related with YRPW motif protein 1 (*HEY1*) and *HEY2*.[57,58] These genes are implicated in vascular development.[57,58] Accordingly, clinical studies have demonstrated downregulation of Notch, HEY, and JAG mRNA expression in placentas of women with preeclampsia.[59,60] Furthermore, Notch signaling in the endocardium regulates cardiac differentiation and coronary vessel development.[61] Our results also demonstrated differential methylation of the Notch signaling pathway in the cord blood of patients with preeclampsia.

We found enrichment of *WWTR1*, which is a downstream effector in the HIPPO pathway, with an important role in cardiac development.[62] The pathway regulates cardiomyocyte proliferation and regeneration, the embryonic knock-out of which causes cardiomegaly

and failure of cardiomyocyte ischemia-reperfusion stress response. Additionally, the HIPPO pathway may directly alter the extracellular matrix composition, with an indirect effect on cardiomyocyte growth, differentiation, and mechanical stress responses.[62]

Additional research is essential to elucidate the connection between these epigenetic alterations and the long-term cardiovascular morbidity in both children and mothers exposed to preeclampsia. If such a link exists, further investigations are necessary to gain a deeper understanding of the underlying pathobiology and develop potential preventive measures.

The findings of this study should be interpreted considering some strength and limitations. While our post-hoc study exceeded the minimum recommended sample size for differential analysis of methylation data obtained from the Illumina 450k beadchip,[63] yielding satisfactory power, we recognize that a larger sample size and more subjects with preeclampsia would have been advantageous. Such an expansion could have enhanced statistical power further, potentially uncovering additional methylation marks, shedding more light on relevant biological pathways, and refining the precision of our estimates regarding methylation differences. Nevertheless, we used all available samples that passed quality control and we used gene expression data from the same subjects to validate our findings, as well as a meta-analysis of several independent datasets as a replication dataset. Also, our dataset consisted of almost exclusively mild preeclampsia cases, limiting the analysis to this specific phenotype. In VDAART, the study participants with existing hypertensive disorders were excluded, removing maternal baseline hypertension as a confounding factor in the cardiovascular risk profile of the offspring. Paternal cardiovascular risk factors, however, should be further investigated for an impact on the cord blood epigenome[64] as a potential confounding factor for cardiovascular disease in the offspring. Human umbilical venous endothelial cells (HUVECs) have been mainly used as an in vitro model for the study of endothelial cell function. Preeclampsia has been shown to affect endothelial function in HUVECs.[65] In freshly isolated primary HUVECs, preeclampsia-dysregulated genes have also shown to be associated with pathobiological pathways in CVD and endothelial function such as nitric oxide synthase signaling.[66] However, venous endothelial cells are phenotypically different from arterial endothelial cells and their dysfunction is linked to cardiovascular disease. Epigenetic mechanisms can contribute to an increased risk of CVD that has its origins in early life.[67] Such epigenetic marks could be modified throughout life by environmental exposures. Longitudinal investigations are needed to determine whether early-life epigenetic alterations persist over time and accumulate in exposure to environmental factors to increase the incidence of CVD. More mechanistic models are required to investigate cellular defects in preeclampsia.[68]

While the VDAART protocol was designed to ensure the purity and integrity of the collected code blood sample, minimizing the risk of maternal contamination, it is important to acknowledge that complete elimination of contamination could be not guaranteed. As newer and potentially more precise methodologies may emerge over time, some methods have presently been developed to address cell mixture distribution and potential maternal blood contamination in cord blood methylation.[24,69,70] The accuracy of these methods, including the deconvolution algorithm used in this study,[24] can be influenced by the quality of the methylation data, the composition of the samples, and the specific algorithm employed. Consequently, residual confounding may not be eliminated entirely. Also, given the current study's design and absence of long-term follow-up of both mothers and children, the longevity of the methylation marks as the children continue to develop remains uncertain. These considerations could be most effectively addressed through a prospective investigation evaluating both maternal and offspring epigenetic modifications in complicated pregnancies with HDP, with a specific focus on assessing their long-term effect on offspring's health. However, there is evidence for the stability of methylation modifications in association with cardiovascular alterations induced by preeclampsia. Brodowski and colleagues showed that DNA methylation of fetal endothelial progenitor cells (ECFCs) at birth was affected by preeclampsia and cell culture passages showed similar gene-network profiles of cardiovascular system development and function.[45] In addition to similarity of the enriched biological pathways with our study, we observed the two constructed networks in these studies are connected and share *EGFR* and *JAG1* as hub genes with the highest degree of connectivity and betweenness centrality. Currently, we do not have blood pressure data from the children in VDAART as they are in early childhood (screening of the pregnant women was done from October 2009 to July 2011) and therefore, we were unable to evaluate the association between cord blood DNA methylation and offspring morbidity. Further follow-up of the cohort is ongoing to definitively determine whether the differential methylation patterns found in this study are related to long-term comorbidities among the offspring of subjects with preeclampsia.

In conclusion, we found that the cord blood of neonates born to pregnant women with preeclampsia shows differential DNA methylation signatures with enrichment in cardiovascular pathways, most notably the apelin signaling pathway. This enrichment was also evident in the gene expression signatures of these subjects or previous reports on the differential methylation in preeclampsia. The differential cord blood DNA methylation signatures should be assessed for stability and in association with long-term morbidity of subjects exposed to preeclampsia.

Contributors

Funding: AL, STW. Concept and design: Input from all authors. Data collection and verification: HMK, PK, HM. Data analysis: HMK, PK, ILS, HM. Statistical support: Input from all authors. Manuscript writing and editing: HMK, ILS and HM with input from all authors. Manuscript approval: All authors read and approved the final manuscript. Agree to be accountable for all aspects of the work: All authors. Corresponding author: Correspondence to Dr. Hooman Mirzakhani.

Data sharing statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

AAL reports personal fees from UpToDate, Inc., outside the submitted work. STW reports income from UpToDate, outside the submitted work. The remaining authors declare no conflicts of interest.

Acknowledgements

We wish to thank all the participants and study staff involved in the Vitamin D Antenatal Asthma Reduction Trial. The Vitamin D Antenatal Asthma Reduction Trial (VDAART) was funded by the National Heart, Lung, and Blood Institute (U03 HL091528 and R01 HL091528 to Drs. Weiss and Litonjua). Dr. Mirzakhani has received research support from NHLBI (U03 HL091528, and 1 K01HL146977 01A1). Dr. Lee-Sarwar has received research support from NHLBI (KO8 332 HL148178).

Funding

VDAART is funded by National Heart Lung and Blood Institute grants of R01HL091528 and UH3OD023268. HMK is supported by Jane and Aatos Erkko Foundation, Paulo Foundation, and the Pediatric Research Foundation. HM is supported by K01 award from NHLBI (1K01HL146977-01A1). PK is supported by K99HL159234 from NIH/NHLBI.

Trial registration

This study is an ancillary analysis from the VDAART, which is registered with ClinicalTrials.gov (NCT00902621).

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