SARS-CoV-2 Covid-19 Infection During Pregnancy and Differential DNA Methylation in Human Cord Blood Cells From Term Neonates

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ABSTRACT

BACKGROUND: The global pandemic of coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). About 18.4% of total Covid-19 cases were reported in children. Even though vertical transmission from mother to infant is likely to occur at a low rate, exposure to COVID-19 during fetal life may alter DNA methylation patterns with potential long-term effects.

OBJECTIVE: To determine if COVID-19 infection during pregnancy alters the DNA methylation patterns in umbilical cord blood cells from term infants and to identify potential pathways and genes affected by exposure to COVID-19 infection.

METHODS: Umbilical cord blood was collected from 8 infants exposed to COVID-19 during pregnancy and 8 control infants with no COVID-19 exposure. Genomic DNA was isolated from umbilical cord blood cells and genome-wide DNA methylation was performed using Illumina Methylation EPIC Array.

RESULTS: 119 differentially methylated loci were identified at the FDR level of 0.20 (64 hypermethylated loci and 55 hypomethylated loci) in umbilical cord blood cells of COVID-19 exposed neonates compared to the control group. Important canonical pathways identified by Ingenuity Pathway Analysis (IPA) were related to stress response (corticotropin releasing hormone signaling, glucocorticoid receptor signaling, and oxytocin in brain signaling pathway), and cardiovascular disease and development (nitric oxide signaling in the cardiovascular system, apelin cardiomyocyte signaling pathways, factors promoting cardiogenesis, and renin-angiotensin signaling). The genes affected by the differential methylations were associated with cardiac, renal, hepatic, neurological diseases, developmental and immunological disorders.

CONCLUSIONS: COVID-19 induces differential DNA methylation in umbilical cord blood cells. The differentially methylated genes may contribute to hepatic, renal, cardiac, developmental and immunological disorders in offspring born to mothers with COVID-19 infection during pregnancy, and their developmental regulation.

KEYWORDS: Global DNA methylation, perinatal COVID-19, SARS-CoV-2, children, epigenetics, cord blood

Introduction

The United States (U.S.) has had over 1 million total deaths attributed to coronavirus disease (SARS-CoV-2) 2019 (COVID-19) since the beginning of the pandemic.[1] This is partly due to the highly infectious nature of SARS-CoV-2 compared to other coronavirus strains. The spike protein subunits S1 and S2, allow for the highly effective binding between the virus and host cells, after which the virus uses the host's splicing machinery for its replication.[2] Even after widespread infection and now vaccination, it is likely that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) will remain present and become endemic in our world.[3] The pediatric population has also experienced a significant death toll as complications like multisystem inflammatory syndrome in children (MIS-C) have been well-documented.[4] Neonates are a vulnerable subgroup with a pressing need for a more detailed understanding of the effects of COVID-19.

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While vertical transmission from mother to infant is likely to occur at a low rate,[5] active disease in neonates can still be severe.[6] The recent multinational study, INTERCOVID, described an increased rate of fetal distress, preterm birth, and lower birth weight in mothers with COVID-19 compared to controls.[7] Adverse perinatal outcomes from maternal COVID exposure likely relates to placental pathological thrombotic, vascular, and inflammatory changes documented in the setting of maternal COVID infection.[8-11] Maternal systemic inflammatory response as well as inflammatory changes in the placenta can provoke a fetal inflammatory response, immune dysregulation, and epigenetic changes that could potentially have long-term consequences in offspring.[7] More recent studies have shown that COVID-19 infection during pregnancy can have an impact on the neonatal immune system affecting T cells and inducing unique inflammatory responses in the maternal-fetal environment.[12,13] We also reported differential gene expression in umbilical cord blood (UCB) of infants exposed to COVID-19 infection during pregnancy.[14] These effects could have long-term impacts on the lives of offspring exposed to this environment, as has been historically seen with life-course studies from those exposed in-utero during the 1918 influenza pandemic.[15]

The link between the perinatal environment and epigenetic changes that may affect disease has been documented in several studies.[16,17] DNA methylation is one mechanism by which this change may occur.[18] It is an enzyme-mediated change during which a methyl group is added to cytosine at specific sites that leads to transcriptional regulation.[19] Intrauterine infections, such as histologic chorioamnionitis, have been associated with epigenetic changes via DNA methylation of neonatal genes.[18,20] Exposure to COVID-19 during pregnancy is likely to have a similar effect in the perinatal environment that could lead to alteration in fetal DNA methylation. We hypothesized that early epigenetic signatures of COVID-19 exposure during pregnancy are detectable in UCB cells. The detection of DNA methylation changes at birth will help to identify at-risk offspring that can benefit from close monitoring and follow-up. Our objective for this study was to evaluate the DNA methylation patterns of human UCB cells from neonates exposed to COVID-19 in-utero and compare those with controls to better understand the epigenetic mechanisms associated with exposure to this virus during pregnancy and their developmental regulation.

Methods

Thomas Jefferson University Institutional Review Board (IRB) approved the study. The details of study design, informed consents, UCB sample collection, processing, storage, DNA isolation and global DNA methylation analysis is described elsewhere.[14,18] In brief, UCB samples were collected from 8 infants exposed to COVID-19 infection during pregnancy and 8 control infants. The genome-wide DNA methylation study was performed using the Illumina Methylation EPIC Array (cat# WG-317-1001, Illumina Inc., San Diego, CA). DNA methylation loci with maximum beta values less than 0.001 across all the 16 samples (223 loci out of 865 918 loci) were filtered out from downstream analyses. Quantile normalization was used to normalize the beta values of remaining loci (865 695 loci).[21] Empirical Bayes method from the Limma package in statistical analysis software R version 4.1.2 was used for comparing the DNA methylation level differences between the Control group and the Covid-19 group.[22] The Benjamini and Hochberg method[23] was used to adjust the raw p-values to control for the false discovery rate (FDR) at 20%. We identified 119 DNA methylation loci at the FDR level of 0.20. Volcano plot was used to examine the association of fold change on log2 scale and the adjusted p-values. Heatmap was used to show the DNA methylation levels of significant DNA methylation loci. The clustering of DNA methylation loci and the samples were based on the ward.D2 clustering method according to Euclidean distance.

Results

Sixteen infants (8 infants COVID-19 group, 8 infants Control group) included in the study and genome-wide DNA methylation was performed on UCB DNA. Eight infants born either before the pandemic (n = 6) or maternal COVID-19 antibody negative at the time of delivery (n = 2) were included in the Control group. One mother was infected during the first trimester, 3 mothers during the second trimester and 4 mothers during the third trimester. Two mothers were symptomatic at the time of delivery, one with severe infection. None of the mothers in Control and COVID-19 groups received COVID-19 vaccine before the sample collection. All 16 infants were healthy at birth, there were no significant differences in mean birth weight (3.14 ± 0.63 kg vs 2.98 ± 0.56, *P* = .6), mean gestational age (38.1 ± 1.3 vs 38.5 ± 1.4, *P* = .6) and other demographic and clinic characteristics in 2 groups described in detail elsewhere.[14]

Differential DNA methylation

DNA methylation levels are measured by β-values which is the ratio of the methylated probe signal intensity to the total locus intensity. The β-values range from 0 (unmethylated) to 1 (fully methylated). DNA methylation loci with maximum beta values less than 0.001 across all the 16 samples (223 loci out of 865 918 loci) were filtered out from downstream analyses. The beta values of remaining loci (865 695 loci) were normalized using quantile normalization method. A total of 119 differentially methylated loci were identified, 64 hypermethylated loci (40 annotated genes) and 55 hypomethylated loci (38 annotated genes) (Supplemental Table 1). The top 20 hypo and hypermethylated gene names and probe-IDs based on p-values are listed in Tables 1 and 2. A volcano plot (Figure 1) was used to examine the association of fold change on log2 scale and the adjusted *P*-values.

Table 1. Top 20 hypermethylated CpGs associated with Covid-19 infection during pregnancy.

TARGET ID	ADDRESS A ID	GENOME BUILD	REFERENCE GENE NAME	LOG FOLD CHANGE	*T*-STATISTICS	*P*-VALUE
cg24801716	81747151	37	XYLB	0.0519	9.048	1.46E-07
cg14362073	16738900	37	TBC1D9B	0.0623	8.195	5.25E-07
cg01270221	7662139	37	SCD	0.0398	8.119	5.91E-07
cg06080729	98797987	37	CLDN6	0.0483	7.725	1.10E-06
cg10547050	70623213	37	PHF12	0.0498	6.987	3.75E-06
cg10308265	4802386	37	UBTF	0.0729	6.918	4.22E-06
cg14002578	20636569	37	GALNT12	0.0793	6.712	6.03E-06
cg10463831	92740155	37	KIF17	0.0390	6.701	6.15E-06
cg05409441	4617426	37	WDR90	0.0491	6.701	6.15E-06
cg05473018	60616834	37	SAMD14	0.0561	6.674	6.45E-06
cg13422555	49738919	37	CLK2	0.0321	6.649	6.73E-06
cg03347381	26665135	37	KIAA0226	0.0515	6.585	7.53E-06
cg01998785	5793285	37	LPCAT2	0.0788	6.577	7.64E-06
cg00688330	41771897	37	HJURP	0.0509	6.488	8.94E-06
cg13208845	88603875	37	KNDC1	0.0271	6.478	9.10E-06
cg11410237	87781944	37	STRN4	0.0790	6.405	1.04E-05
cg25052933	65752852	37	HLA-DPB1	0.0610	6.345	1.15E-05
cg07262630	70750521	37	PLEC	0.0525	6.274	1.31E-05
cg15985126	23687174	37	SAR1B	0.0661	6.259	1.35E-05
cg08648882	82735379	37	ENSA	0.0526	6.253	1.36E-05

Cluster analysis—Heatmap

Hierarchical clustering method was used to perform the cluster analysis on the 119 differentially methylated loci for the 2 groups. Heatmap was used to show the DNA methylation levels of significant DNA methylation loci (Figure 2). The clustering of DNA methylation loci and the samples were based on the ward.D2 clustering method according to Euclidean distance.

Ingenuity pathway analysis

Ingenuity Pathway Analysis (IPA) software (https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis, Qiagen Inc., Germantown, MD)[24] was used for pathway analysis by loading 412 probe sets (290 annotated genes) that were differentially methylated with exposure to Covid-19 infection (*P*-value ≤ .0001). A total of 340 canonical pathways were altered with exposure to Covid-19 infection during pregnancy. Selected key pathways important in Covid-19 pathophysiological response are shown in Table 3. Seventy-six diseases and functions were modified with Covid-19 exposure. Selected key diseases and functions altered with Covid-19 exposure are listed in Table 4.

Tox functions

COVID-19 exposure during pregnancy potentially altered 26 tox functions in UCB (Table 5). Top tox functions related to cardiotoxicity include cardiac arteriopathy, cardiac stenosis, congenital heart anomaly. Top altered hepatotoxic functions are liver hyperbilirubinemia, liver proliferation, liver inflammation/hepatitis, liver cirrhosis, liver fibrosis, liver hyperplasia/hyperproliferation, and liver cholestasis. Top altered tox functions related to nephrotoxicity are nephrosis, renal dysplasia, renal hypoplasia, renal necrosis/cell death, renal proliferation, glomerular injury, renal inflammation, renal nephritis.

Upstream regulators

Upstream regulator analysis by IPA identified 72 regulators to be dysregulated. Key identified regulators and their target molecules are described in Table 6.

Table 2. Top 20 hypomethylated CpGs associated with Covid-19 infection during pregnancy.

| TARGET ID	ADDRESS A ID	GENOME BUILD	UCSC REFERENCE GENE NAME	LOG FOLD CHANGE	*T*-STATISTICS	*P*-VALUE
cg20336007	65615162	37	BMP7	−0.5219	−24.259	1.05E-13
cg17377263	44735271	37	RHOBTB1	−0.0895	−9.210	1.16E-07
cg10000867	14737981	37	CFAP69	−0.1134	−7.550	1.47E-06
cg26700469	3675495	37	WWOX	−0.1146	−7.360	2.00E-06
cg27134827	43688855	37	PPT2	−0.0345	−7.039	3.43E-06
cg14515812	40701105	37	RAP1GAP	−0.0320	−6.952	3.98E-06
cg25587223	45650134	37	GUCY2D	−0.0279	−6.816	5.03E-06
cg22931151	6643305	37	OR2V2	−0.4824	−6.718	5.97E-06
cg11275630	19766117	37	SLC8A2	−0.1028	−6.708	6.08E-06
cg17431391	56774849	37	ZNF540	−0.0674	−6.639	6.85E-06
cg03477130	20740100	37	HLA-DOB	−0.0587	−6.566	7.80E-06
cg14119337	85685313	37	MEG3	−0.0689	−6.540	8.15E-06
cg02931058	76603468	37	KLHL30	−0.1105	−6.263	1.34E-05
cg20391833	42771267	37	RPS6KA2	−0.0791	−6.219	1.45E-05
cg25150313	95731891	37	CDH23	−0.0621	−6.158	1.62E-05
cg20952167	70682845	37	MEG3; MEG3; MEG3	−0.0647	−6.141	1.67E-05
cg11315777	79690504	37	MAP3K7CL	−0.0726	−6.140	1.67E-05
cg26314066	87614203	37	CNTD1	−0.0418	−6.132	1.70E-05
cg12590430	2622447	37	AMPH	−0.0578	−6.122	1.73E-05
cg10440011	78779322	37	DPH1	−0.0840	−6.100	1.80E-05

Discussion

In this observational and hypothesis generating study, we report that COVID-19 infection during pregnancy is associated with differential DNA methylation in UCB cells. Exposure to COVID-19 infection during pregnancy altered the fetal patterns of DNA methylation in genes related to stress response (corticotropin releasing hormone signaling, glucocorticoid receptor signaling, and oxytocin in brain signaling pathway), and cardiovascular disease and development (nitric oxide signaling in the cardiovascular system, factors promoting cardiogenesis, and apelin cardiomyocyte signaling pathways, and renin-angiotensin signaling). The genes affected by the differential methylations were associated with cardiac, renal, hepatic, neurological diseases, developmental and immunological disorders. The genes involved with dysregulated methylation were also associated with cardiac, hepatic, and renal toxicity and key up-stream regulators.

The top hypomethylated genes included BMP7 (Bone morphogenetic protein 7), RHOBTB1 (Rho related BTB domain containing 1), WWOX (WW domain containing oxidoreductase), and HLA-DOB (Major histocompatibility complex, class II, DO beta). BMP7 codes a ligand of TGF-beta (transforming growth factor-beta) that binds certain TGF-beta receptors and leads to recruitment and activation of SMAD family transcription factors. BMP7 is associated with bone disorders, renal injury, and pulmonary fibrosis.[25-27] Progressive pulmonary fibrosis (lung remodeling) is thought to lead to higher mortality in COVID-19 patients. Severity of disease is associated with activation of the TGF-beta pathways that triggered inflammation, apoptosis, and fibrosis. The BMP7 pathway has been shown to oppose the negative effects of TGF-beta, blocking inflammation and apoptosis and reversing fibrosis.[28] Hypomethylation of BMP7 gene if persisted later in life can potentially reduce the risk of lung fibrosis in offspring. WWOX encodes a member of short-chain dehydrogenases/reductases protein family and appears to function as tumor suppressor gene. In a recent study, Dugan et al demonstrated that the genetic locus containing the WWOX gene was implicated as a clinical Alzheimer's disease risk locus.[29] In 2019, a study reported the vital implications of WWOX in atherosclerosis and cardiovascular diseases.[30] Another study has shown that inhibition of WWOX, contributes to pulmonary remodeling and subsequent pulmonary arterial hypertension.[31]
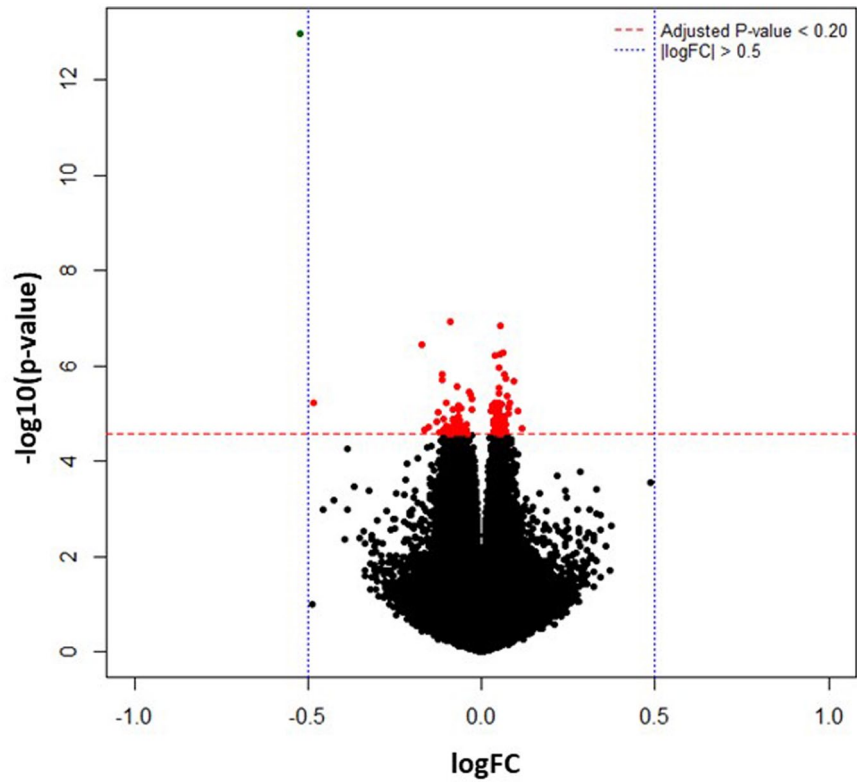
Figure 1. The differentially methylated CpG sites between the COVID-19 and the Control groups are depicted in this volcano plot. The x-axis shows difference in mean percent of DNA methylation and the y-axis shows the significance of differential methylation of each probe, represented as the negative log of the p-value. The red horizontal line indicates genome-wide significance threshold of FDR ≤ 0.20. The blue vertical line signifies |Log Fold Change| > 0.05.
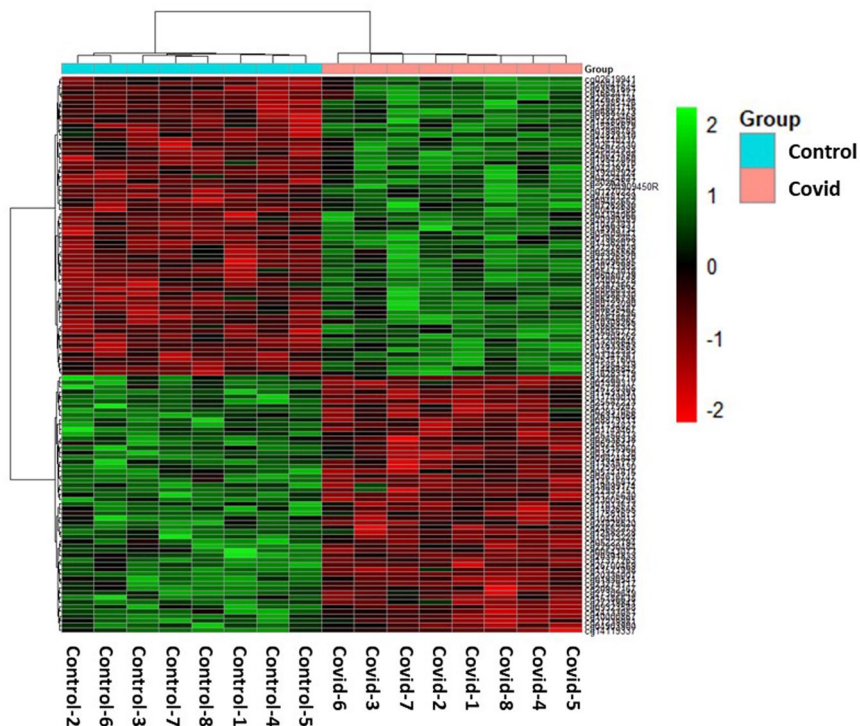
Figure 2. The 119 differentially methylated probe sets (FDR *P*-value ≤ .20)) in the individual Covid-19 samples (right) compared to Control samples (left). The green and red colors indicate hyper-methylated and hypo-methylated loci, respectively.

Table 3. Canonical pathways picked-up by ingenuity pathway analysis of the differentially methylated genes in Covid-19 group compared to the Control group.

INGENUITY CANONICAL PATHWAYS	-LOG (*P*-VALUE)	GENES INVOLVED IN PATHWAYS	IMPORTANCE
Nitric Oxide Signaling in the Cardiovascular System	3.95	CACNG8,GUCY2D,ITPR2, NOS3,PIK3CB,PRKCE	Cardiovascular and immune systems
Corticotropin Releasing Hormone Signaling	3.35	CACNG8,CREB5,GUCY2D, ITPR2,NOS3,PRKCE	Pituitary gland, stress response
Factors Promoting Cardiogenesis in Vertebrates	3.34	BMP3,BMP7,CER1,CREB5, PRKCE,WNT6	Formation of heart in neonates
Apelin Cardiomyocyte Signaling Pathway	3.31	MAPK6,NOS3,PIK3CB, PRKCE,SLC8A2	Cardiovascular diseases, Obesity and Cancer
Oxytocin Signaling Pathway	3.3	CACNG8,CREB5,GUCY2D, ITPR2,MAPK6,NOS3, PIK3CB,PRKCE	Promotes lactation, involved in reproductive and social behavior
Growth Hormone Signaling	2.9	IGF1R,PIK3CB,PRKCE, RPS6KA2	Stimulate the pituitary gland, control metabolism and growth
Estrogen Receptor Signaling	2.85	CACNG8,CREB5,FOXA1, IGF1R,NOS3,PIK3CB, PPARGC1A,PRKCE,TRRAP	Promotes sexual organ maturation and development in female
Endothelin-1 Signaling	2.82	GUCY2D,ITPR2,MAPK6, NOS3,PIK3CB,PRKCE	Vasoconstrictive action, causes fibrosis of the vascular cells
Synaptic Long Term Depression	2.8	CACNG8,GUCY2D,IGF1R, ITPR2,NOS3,PRKCE	Weakens synapses efficiency
Adrenomedullin signaling pathway	2.73	GPR182,GUCY2D,ITPR2, MAPK6,NOS3,PIK3CB	Regulation of endothelial barrier function, circulation control, vasodilation
Insulin Secretion Signaling Pathway	2.69	CACNG8,CREB5,ITPR2,PCSK2,PIK3CB,PRKCE,SEC11A	Crucial role on cell metabolism, growth, proliferation and differentiation
Opioid Signaling Pathway	2.6	CACNG8,CREB5,ITPR2, MAPK6,NOS3,PRKCE, RPS6KA2	Mediates analgesic effect
Role of NFAT in Cardiac Hypertrophy	2.47	CACNG8,IGF1R,ITPR2, PIK3CB,PRKCE,SLC8A2	Development of the cardiovascular system, Embryonic heart
eNOS Signaling	2.47	HSPA4,ITPR2,NOS3, PIK3CB,PRKCE	Vascular homeostasis, contributes to angiogenesis
AMPK Signaling	2.25	CREB5,NOS3,PIK3CB,PPARGC1A, PPM1H,SMARCE1	Regulation of cellular energy homeostasis
Renin-Angiotensin Signaling	2.07	ITPR2,PAK2,PIK3CB,PRKCE	Controls blood pressure and sodium homeostasis, critical regulator of blood volume and systemic vascular resistance
Oxytocin In Brain Signaling Pathway	2.03	CACNG8,CREB5,ITPR2, PIK3CB,PRKCE	Significant roles in heart and brain, regulates hypothalamo-pituitary-adrenal axis, modulating behavioral response toward stress and social behavior
FXR/RXR Activation	2.02	FOXA1,PPARGC1A,RBP4, SLCO1B3	Alters expression of different groups of genes involved in bile acid homeostasis, lipid metabolism, and glucose balance
Angiopoietin Signaling	1.85	NOS3,PAK2,PIK3CB	Involved in vascular development and stability, angiogenesis and quiescence
Glucocorticoid Receptor Signaling	1.56	DNAH10,HLA-DOB,HLA-DPB1, HSPA4,IL1R2,NOS3, PIK3CB,SMARCE1	plays essential role in the cardiovascular system
Circadian Rhythm Signaling	1.51	CACNG8,CREB5,GUCY2D, ITPR2,PRKCE	regulates the circadian rhythm of a cell or organism, senses external timing signals like light/dark
Th1 Pathway	1.37	HLA-DOB, HLA-DPB1, PIK3CB	Mediates cellular response to infection, identifies and eradicates of intracellular pathogens such as viruses and bacteria

Table 4. Modified functions and diseases obtained by differentially methylated genes after exposure to Covid-19 infection during pregnancy.

MODIFIED DISEASES AND FUNCTIONS	RANGE OF *P*-VALUES FOR GENES INVOLVED	NUMBER OF GENES INVOLVED
Cancer	3.98E-07-3.9E-02	138
Organismal Injury and Abnormalities	3.98E-07-3.9E-02	140
Dermatological Diseases and Conditions	9.24E-06-1.97E-02	108
Gastrointestinal Disease	2.82E-05-3.72E-02	128
Cell Morphology	1.3E-04-3.9E-02	17
Hepatic System Disease	1.8E-04-1.97E-02	83
Reproductive System Disease	3.27E-04-3.9E-02	95
Cellular Development	4.28E-04-3.83E-02	24
Connective Tissue Development and Function	4.28E-04-3.9E-02	10
Tissue Morphology	4.28E-04-3.9E-02	15
Endocrine System Disorders	5.23E-04-3.9E-02	118
Embryonic Development	6.4E-04-3.26E-02	12
Organismal Development	6.4E-04-3.9E-02	17
Cellular Growth and Proliferation	7.14E-04-3.9E-02	24
Nervous System Development and Function	7.14E-04-3.83E-02	9
Neurological Disease	8.91E-04-3.9E-02	92
Developmental Disorder	1.03E-03-3.9E-02	29
Renal and Urological Disease	1.03E-03-3.63E-02	14
Cardiovascular Disease	1.4E-03-2.36E-02	5
Cell-To-Cell Signaling and Interaction	1.91E-03-3.9E-02	16
Hematological System Development and Function	1.91E-03-3.9E-02	14
Immunological Disease	3.23E-03-3.61E-02	43
Respiratory Disease	5.78E-03-3.9E-02	63

In a gene ontology study on Covid-19 patients with respiratory failure, Oh and coworkers identified WWOX to be one of the 8 genes from the largest connected network.[32] HLA-DOB suppresses peptide loading of MHC class II molecules. Recently, HLA-DOB was observed to be one of the 13 key differentially expressed genes in preterm infants who developed bronchopulmonary dysplasia (BPD) and expression was increased in infants with BPD.[33] Due to hypomethylation of HLA-DOB CpGs with COVID-19 infection in pregnancy, expression of HLA-DOB is likely to be increased in preterm infants and could potentially increase the risk of BPD.

The top hypermethylated genes included XYLB (Xylulose kinase), CLDN6 (Claudin-6), UBTF (Upstream binding transcription factor), LPCAT2 (Lysophosphatidylcholine acyl-transferase 2), STRN4 (Striatin 4), and HLA-DPB1 (Major histocompatibility complex, class II, DP beta 1). XYLB plays an important role in the regulation of glucose metabolism and lipogenesis. Decreased plasma product from XYLB was hypothesized as one of the potential metabolomics biomarkers of COVID-19.[34-36] CLDN6 (Claudin-6) has a large role in tight junction-specific obliteration of the intercellular space (which has a role in microbial infection), and also functions as a receptor for hepatitis C virus entry into hepatic cells. UBTF gene is associated with neurodegeneration, brain atrophy, intellectual disability, retinoblastoma, pediatric acute myeloblastic leukemia.[37,38] Integrative network analysis in transcriptional landscape of circulating platelets from COVID-19 patients identified downregulated UBTF as a key factor for disease severity. Abate et al. has shown that LPCAT2 co-localizes with TLR4 and modulates macrophage inflammatory gene expression in response to lipopolysaccharide.[39] In another study LPCAT2 methylation was identified to be a biomarker for cedar pollen allergic rhinitis severity in the Japanese population.[40] STRN4 (Striatin 4) is associated with muscular

Table 5. Top tox functions modified with Covid-19 infection during pregnancy.

MODIFIED TOX FUNCTIONS	CATEGORY	RANGE OF *P*-VALUES FOR GENES INVOLVED	NUMBER OF MOLECULES
Cardiac Arteriopathy	**Cardiotoxicity**	6.61E-03-2.97E-01	4
Cardiac Stenosis		8.26E-02-8.26E-02	1
Congenital Heart Anomaly		1.01E-01-4.07E-01	2
Liver Hyperplasia/Hyperproliferation	**Hepatotoxicity**	2.65E-04-5.5E-01	74
Liver Hyperbilirubinemia		1.97E-02-1.97E-02	1
Liver Proliferation		8.87E-02-8.87E-02	1
Liver Inflammation/Hepatitis		2.2E-01-4.35E-01	2
Liver Cirrhosis		2.43E-01-5.28E-01	3
Liver Fibrosis		2.43E-01-5.28E-01	3
Liver Cholestasis		3.33E-01-3.33E-01	1
Nephrosis	**Nephrotoxicity**	6.61E-03-6.61E-03	1
Renal Dysplasia		6.61E-03-7.62E-03	2
Renal Hypoplasia		6.61E-03-6.61E-03	1
Renal Necrosis/Cell Death		3.3E-02-5.41E-01	7
Renal Proliferation		1.44E-01-1.44E-01	3
Glomerular Injury		2.23E-01-5.44E-01	2
Renal Inflammation		2.23E-01-5.44E-01	2
Renal Nephritis		2.23E-01-5.44E-01	2

**Table 6.** Key upstream regulators dysregulated with Covid-19 exposure during pregnancy.

UPSTREAM REGULATORS	MOLECULE TYPE	*P*-VALUE OF OVERLAP	TARGET MOLECULES IN DATASET
LPIN1	phosphatase	.000441	LPIN1,PPARGC1A
KLF5	transcription regulator	.000508	CAVIN1,FOXA1,NMI,PTGES
AREG	growth factor	.000851	CCNF,HJURP,KIF20A,SSRP1
MITF	transcription regulator	.00112	CCNF,KIF20A,KIFC1,PPARGC1A,SCD
HCAR2	G-protein coupled receptor	.00282	KIF20A,NMI
mir-218	microRNA	.00443	MDGA1,SASH1
EPAS1	transcription regulator	.0055	HSPA4,LOX,NEK8,NOS3
NPC2	transporter	.00637	CREB5,PTGES
Histone h3	group	.00784	FOXA1,GBX2,IGF1R,NOS3,PTGES,SCD
EZH2	transcription regulator	.0125	BMP7,CER1,FOXA1,mir-10,PTGES

dystrophy, cerebral cavernous malformations and cancer. Chakraborty et al and coworker have reported upregulation of STRN4 gene expression with Sars-Cov-2 infection.[41]

We compared the differentially methylated loci with our recently published gene expression data.[14] Four genes are common for both DNA methylation and gene expression data. Two differentially methylated genes had reciprocal changes in gene expression. XYLB is hypermethylated with reciprocal down-regulated gene expression and ras-associated protein-1 GTPase-activating-protein (RAP1GAP) is hypomethylated with reciprocal upregulated gene expression. In animal models, RAP1GAP inhibit autophagy, increase oxidative stress and
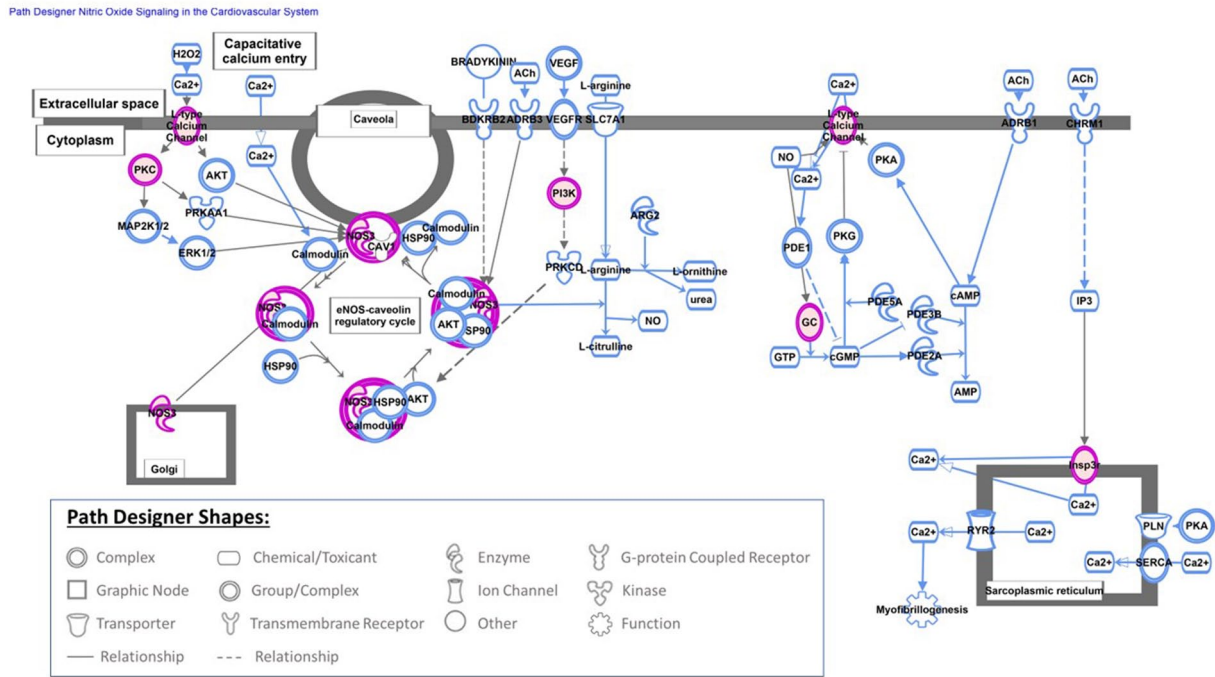
Figure 3. Nitric oxide (NO) signaling in the cardiovascular system is shown in this figure which is the top-most canonical pathway picked up by Ingenuity Pathway Analysis (IPA) software (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA) by loading 412 probe sets (290 annotated genes) that were found to be differentially methylated with exposure to COVID-19, *P*-value ≤ .0001.

mediates angiotensin II-induced cardiomyocyte hypertrophy.[42] Two other genes, HLA-DPB1 and ENSA have methylation and gene expression in the same direction (hypermethylated loci and upregulated gene expression).

Altered methylation pattern in genes involved in canonical pathways can potentially link COVID-19 exposure during pregnancy to short- and long-term outcomes in offspring. At least, 340 canonical pathways were found to be altered in UCB cells exposed to COVID-19 infection during pregnancy. COVID-19 infection as well as stress exposure may alter patterns of DNA methylation in genes related to stress. Altered DNA methylation status of stress-related genes have been reported as the epigenetic changes due to adverse events during early life including fetal period.[43-45] Our data also indicates that infection with COVID-19 in pregnancy altered the fetal patterns of DNA methylation in genes related to the stress response. The methylation of genes involved in corticotropin releasing hormone signaling, glucocorticoid receptor signaling, opioid signaling pathway and oxytocin in brain signaling pathway were affected by the exposure to COVID-19 infection. Persistence of altered DNA methylation in genes related to stress can potentially affect child development and increase the risk for behavior and psychological disorders. COVID-19 infection during pregnancy also altered the DNA methylation pattern in genes involved in cardiovascular development and disease. The most altered canonical pathway in our cohort after exposure to COVID-19 infection was nitric oxide (NO) signaling in the cardiovascular system (Figure 3). Nitric oxide is a free radical that plays an important physiological role in

cardiovascular and immune systems.[46] We also observed an altered DNA methylation pattern in genes related to: cardiogenesis in vertebrates, the apelin cardiomyocyte signaling pathway (Figure 4), NFAT (nuclear factor of activated T cells) and cardiac hypertrophy (Figure 5), and the eNOS signaling pathway. The role of NO, apelin, and NFAT have been reported in the pathogenesis of COVID-19 infection.[47] Apelin plays an important role in alleviating Ang-II mediated acute lung and cardiovascular injuries and prothrombotic events in COVID-19 patients.[48] The NFAT (nuclear factor of activated T cells) pathway contributes to the pathogenesis of cardiac hypertrophy and plays a critical role in Ca(2+)/calcineurin-mediated cardiac hypertrophic signaling. Excessive CaN/NFAT activation and TRPC overproduction in the heart contribute to the development and progression of pathological myocardial hypertrophy.[49] The role of altered gene methylation related to these pathways needs further investigation if persistence of altered methylation in infants increases the risk of heart disease later in life.

Exposure to COVID-19 infection during pregnancy altered 26 toxic functions in our cohort. The top toxic functions that altered DNA methylation were seen in genes related to hepatotoxicity, cardiotoxicity, and nephrotoxicity. Cardiac, hepatic and renal dysfunctions are commonly observed in children with MIS-C.[50] Li et al reported that the SARS-CoV-2 virus can shut down energy production in cells of the kidneys, heart, spleen and other organs in an animal model.[51] Additionally, late cardiovascular, liver and renal complications after COVID-19 infection have been reported.[52] Altered DNA methylation
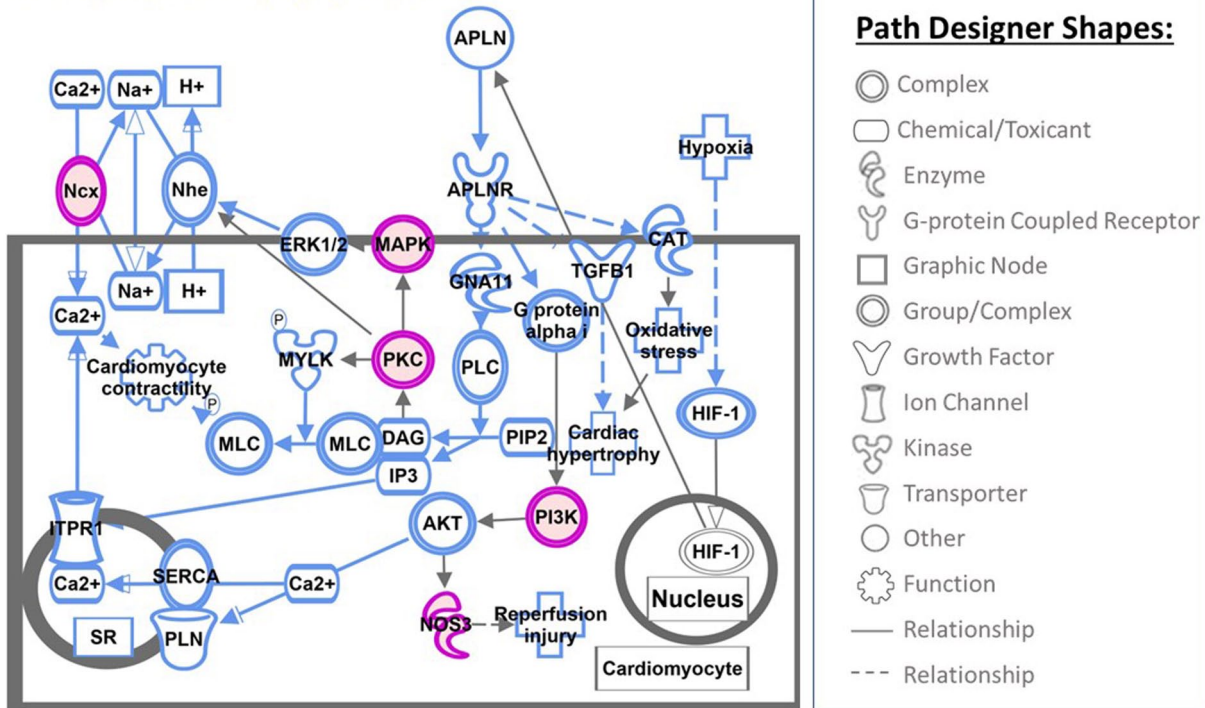
Figure 4. This figure depicts the Apelin cardiomyocyte signaling canonical pathway obtained from IPA pathway analysis. Apelin is an important regulator of cardiac contractility in cardiomyocytes and also protects the heart against cardiac hypertrophy and ischemia-reperfusion injury.
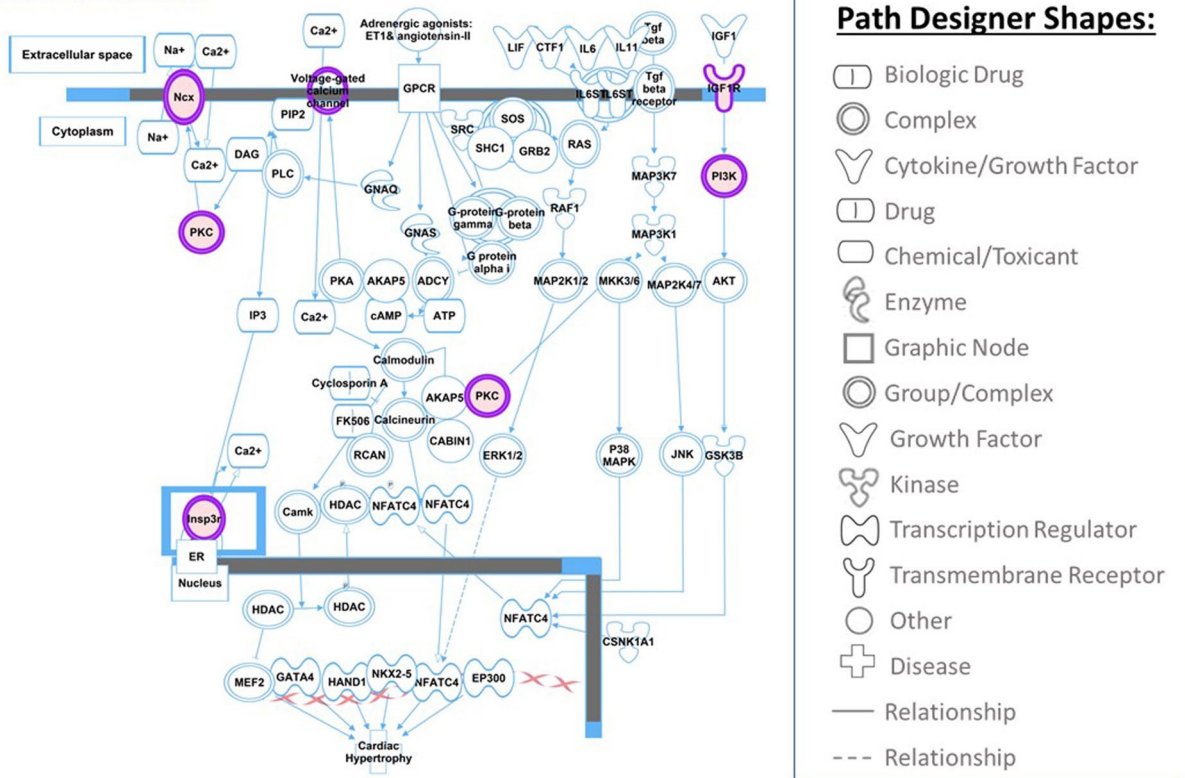
Figure 5. Role of NFAT in Cardiac Hypertrophy pathway is presented in this figure.

in genes related to cardiac, hepatic and renal toxic functions after COVID-19 infection during pregnancy may potentially increase the risk for heart, liver and kidney diseases in offspring and should have longitudinal follow-up.

Upstream regulator analysis by IPA identified 72 regulators to be dysregulated after COVID-19 exposure during pregnancy including Krüppel-like factor 5 (KLF5), melanocyte inducing transcription factor (MITF), endothelial PAS domain protein 1 (EPAS1) and enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2). KLF5 is a zinc finger-type transcription factor and is involved both in promoting and suppressing cell proliferation. KLF5 was associated with severe COVID-19 infection and hospitalization.[53] The diseases associated with KLF5 include: cardiomyopathy, sigmoid disease, familial partial lipodystrophy, arteriosclerosis and pancreatic cancer.[54] MITF plays a role in the development, survival, and function of various types of cells. In melanocytes, this protein controls production of melanin, which contributes to hair, eye, and skin color. Jeong et al identified MITF to be one of the transcription factors that could drive the transcriptomic changes for the pathogenesis of COVID-19 and markers related to its severity.[55] The EPAS1 gene codes for a transcription factor involved in the induction of genes regulated by oxygen, which is induced as hypoxia occurs. EPAS1 regulates vascular endothelial growth factor (VEGF) expression, which is involved in the development of blood vessels and the tubular system of lung. EZH2 provides instructions for making the histone methyltransferase enzyme that can suppress the activity of certain genes via methylation. LIPN1 (Lipin 1) acts as a magnesium-dependent phosphatidate phosphatase enzyme and is involved in adipocyte differentiation. The protein encoded by AREG (ampiregulin) gene is a member of the epidermal growth factor family. Using bulk RNA-sequencing showed an increase of AREG in the peripheral blood monocytes (PBMCs) of COVID-19 patients, supporting a potential role of AREG in SARS-CoV-2-induced lung pathology.[56] Bui et al have reported that AREG is significantly upregulated in PBMCs, CD4T Cells, NK cells, neutrophils, and dendritic cells, suggesting that upregulation of AREG may be an attempt to ameliorate the severe injury induced by SARS-CoV-2 infection.[57] HCAR2 (Hydroxycarboxylic Acid Receptor 2) is a niacin receptor associated with inflammatory bowel disease, diversion colitis and pellagra. NPC2 (NPC Intracellular Cholesterol Transporter 2) is secreted from the liver into bile and plasma, where it may have a functional role in cholesterol transport in physiologic and pathologic conditions.[58] In a 2021 study, Prasad et al reported that NPC2 is one of the genes linked with neurological disease, which is also connected to COVID-19.[59] Histone H3 is one of the 5 main histones involved in the structure of chromatin in eukaryotic cells. Histone H3 plays a crucial role in activating the spindle assembly checkpoint in response to a defect in mitosis. The presence of histone H3 during ICU stays was associated with thromboembolic events and secondary infection.[58] Non-cleaved histone H3 was associated with the need for vasoactive treatment, invasive ventilation, and the development of acute kidney injury in COVID-19 patients.[60] In a review article, Ligi et al. concluded that monitoring systemic histone concentrations (both unmodified and citrullinated histone H3) of patients with SARS-CoV-2 infection throughout their hospitalization (especially in critical patients and/or those needing intensive care) may be a useful tool for early prediction of higher risk of disease progression.[61]

To our knowledge, this is the first study reporting differential DNA methylation in UCB cells from infants exposed to COVID-19 infection during pregnancy. More recently, Hill et.al, reported differential methylation of several genes including genes in pathways that play a role in human neurodevelopment in buccal swabs on infants exposed to in utero COVID-19 infection.[62] The altered methylation pattern identified in genes related to hepatic, renal, and cardiac diseases show a potential connection to long-term consequences of COVID-19 infection. Our study, however, also has several limitations, for example, the sample size of 16 neonates is small, but similar limited sample sizes have been used in studies investigating differential DNA methylation patterns.[63,64] There is an inherent chance of finding differences in the DNA methylation due to multiple comparison and our results should be considered as being hypothesis generating and should be validated in a larger cohort.

In conclusion, SARS-CoV-2 COVID-19 exposure during pregnancy induces differential DNA methylation in UCB cells. We identified 119 differentially methylated genes, important tox functions, upstream regulators and canonical pathways related to the stress response, hepatotoxicity, nephrotoxicity and cardiotoxicity in UCB cells of neonates born to mothers with COVID-19 infection during pregnancy. Future studies can further validate differential methylation of target genes in a larger cohort of infants. Our data may help to further understanding the role of key pathways and genes on the long-term consequences related to COVID-19 infection during pregnancy. Functional studies on the identified pathways and genes could lead to the development of biomarkers for the illnesses in offspring caused COVID-19 infection during pregnancy. Lastly, our results underscore the importance of determining downstream consequences in offspring of mothers who had COVID-19 infection during pregnancy even in the absence of vertical transmission of disease.

Author Contribution Statement

PU, SGB contributed equally to concept and design, sample collection and processing, acquisition and assembly of data, data

analysis and interpretation and manuscript writing and share first authorship. SA contributed to data analysis and interpretation. RSG, JSC, HA, RCB, KS, DL, IR contributed to concept and design, sample collection and processing, and manuscript writing. ZA contributed to concept and design, data analysis and interpretation and manuscript writing. All authors have approved the version of the submitted manuscript.

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Supplemental material
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