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DNA methylation patterns in umbilical cord blood from infants of methadone maintained opioid dependent mothers

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Methadone maintenance treatment for opioid dependent mothers is standard of care. Infants of methadone maintained opioid dependent (MMOD) mothers have better outcomes compared to infants of opioid dependent mothers without treatment. However, when compared to non-exposed infants, infants of MMOD mothers are associated with worse outcomes. We conducted a pilot study to examine genome wide differential DNA methylation using cord blood samples from sixteen term and near-term infants of MMOD and opioid naïve mothers, excluding Infants with chorioamnionitis. A total of 152 differentially methylated loci were identified at a difference > + 2, < − 2 and p-value < 0.05. There were 90 hypermethylated loci (59 annotated genes) and 62 hypomethylated loci (38 annotated genes) observed. The hypermethylated and hypomethylated DNA changes involved multiple genes, pathways and networks that may explain some of the changes seen in infants of MMOD mothers. Top hypermethylated and hypomethylated genes involved areas of cell growth, neurodevelopment, vision and xenobiotic metabolism functions. Our data may explain the role of key pathways and genes relevant to neonatal outcomes seen from methadone exposure in pregnancy. Functional studies on the identified pathways and genes could lead to improved understanding of the mechanisms and identify areas for intervention.

Medication assisted treatment with methadone or buprenorphine is considered standard of care for opioid dependent pregnant persons[1]. When compared to untreated mothers using illicit opioids, methadone maintained opioid dependent (MMOD) mothers have better perinatal outcomes[2]. However, when compared to non-opioid exposed pregnant persons (or individuals), methadone exposure was associated with worse perinatal outcomes including increased risk for shorter gestation periods, lower birth weight, smaller head circumference, increased risk of developing neonatal opioid withdrawal syndrome (NOWS) and impaired visuocortical function at birth[3–5]. Also, children delivered to MMOD pregnant persons were found to exhibit lower psychomotor, cognitive, behavioral and language scores, in follow up studies[6,7]. Some proposed mechanisms by which opioid exposure influenced fetal development include inhibition of neuronal proliferation and differentiation with increased cell death, alterations in endocrine function and modifications to myelin sheath formations[8,9].

DNA methylation is considered an important mechanism for changes seen in the developing fetus in response to medications and stress[10]. This idea is linked to the concept of Developmental Origins of Health and Disease (DOHad) hypothesis; a conceptual framework that links prenatal environmental exposures to subsequent health and disease outcomes later in life[11]. Epigenetic mechanisms, most importantly, changes in DNA methylation, has been suggested as one of the mechanisms of the outcomes seen in infants of MMOD mothers[12]. DNA methylation refers to the addition of a methyl (-CH3) group to the fifth position of cytosine nucleotide in areas of the genome where cytosine is followed immediately by guanine in the DNA sequence referred to as a CpG dinucleotide.

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This leads to the formation of 5-methylcytosine, an enzymatic 1-carbon metabolism process. Hypermethylated regions on genes are associated with decreased expression while hypomethylated regions are associated with increased gene expression[13,14]. Other epigenetic mechanisms like histone protein modification and non-coding RNAs have not been well studied as possible contributors to the associated outcomes.

Whole genome methylation profiling has been extensively utilized to evaluate the epigenetic basis of various human pathology[15]. Genome-wide DNA methylation profiling coupled with artificial intelligence (AI) has been used to create a pathway and network analysis with the aim of diagnosing maternal opioid exposure and predicting infants who will develop NOWS[16]. DNA methylation changes in opioid receptor related genes obtained from buccal samples of methadone-maintained mother and infant dyads found increased methylation in *ABCB1, CYP2D6, and OPRM1* genes but no correlation with outcomes[12].

To date, no study has examined differential DNA methylation regions (DMRs) in genome wide studies in umbilical cord blood (UCB) to form the basis of explaining neonatal outcomes for MMOD pregnant persons. Findings of DMRs in cord blood samples of infants of MMOD mothers may help explain some of the outcomes seen in these infants, especially if the methylation patterns persist and or correlate with gene expression associated with known disease processes. The aim of this pilot study is to determine if maternal methadone maintenance leads to differential neonatal DNA methylation patterns using UCB samples in full term and near-term infants.

We hypothesized that infants of MMOD persons will have differential DNA methylation compared to infants of opioid naïve persons.

Results

In this pilot study, we enrolled 16 infants (8 infants in the Methadone group and 8 infants in the Control group) and performed genome-wide DNA methylation on UCB DNA. At delivery all 16 infants appeared healthy. There were no significant differences in mean birth weight (2.76 ± 0.34 kg vs 3.12 ± 0.44, p = 0.06), mean gestational age (38.8 ± 0.9 vs 38.5 ± 1.5, p = 0.3), exposure to cigarette smoking (4/8 vs 2/8, p = 0.6) and other exposures, between the methadone exposed group and the control (Table 1).

Differential DNA methylation

DNA methylation levels are represented by β-values. The β-value is the ratio of the methylated probe signal intensity to the total locus intensity. The β-values range from 0 to 1 where 0 indicates unmethylated and 1 indicates fully methylated. A boxplot (Fig. 1) was generated from β-values of all probe sets from the 16 samples to describe distribution of the data. No significant differences were observed between the groups. A total of 152 differentially methylated loci were identified at a difference > + 2, < − 2 and p-value < 0.05, of which 90 are hypermethylated loci (59 annotated genes) and 62 are hypomethylated loci (38 annotated genes). The top 20 hyper and hypomethylated gene names and probe-IDs, in the exposure group, based on p-values are listed in Tables 2 and 3.

Cluster analysis: heatmap

Cluster analysis was performed on the 152 differentially methylated loci for the two sample groups using the Heatmap function in Partek Genomics Suite Software. A heatmap of the methylation levels for the 152 DNA methylation loci illustrates the differences between the two groups (Fig. 2).

Ingenuity pathway analysis

Ingenuity Pathway Analysis (IPA) software, Qiagen Inc., Germantown, MD) was used for pathway analysis by loading 152 probe sets (97 annotated genes) that were differentially methylated with exposure to methadone. A total of 325 canonical pathways were altered with exposure to methadone during pregnancy. Selected key pathways important in methadone pathophysiological response are shown in (Table 4, Figs. 3, and 4). Seventy-three

	Methadone n = 8 (%)	Control n = 8 (%)	Total N = 16 (%)	P value
Gestational age	38.8 ± 0.9	38.5 ± 1.5	38.2 ± 1.25	0.29
Sex (Male)	2(25.0)	6 (75.0)	8 (50.0)	0.13
Weight (Kg)	2.76 ± 0.34	3.12 ± 0.44	2.94 ± 0.43	0.06
Cigarette exposure	4 (50.0)	2 (25.0)	6 (37.5)	0.61
Diabetes†	0 (0)	2 (25.0)	2 (12.5)	0.48
Hypertension*	3 (37.5)	1 (12.5)	4 (25.0)	0.57
Other exposures				
Fentanyl	3 (37.5)	0 (0)	3 (18.8)	0.20
Cocaine	3 (37.5)	0 (0)	3 (18.8)	0.20
Clonidine	2 (25.0)	0 (0)	2 (12.5)	0.47
Benzodiazepine	1 (12.5)	0 (0)	1 (6.3)	1.0
Penicillin	1 (12.5)	1 (12.5)	2 (12.5)	1.0

Table 1. Baseline and Clinical Characteristics. †Gestational and pre-gestational diabetes. *Chronic hypertension and Gestational Hypertension.

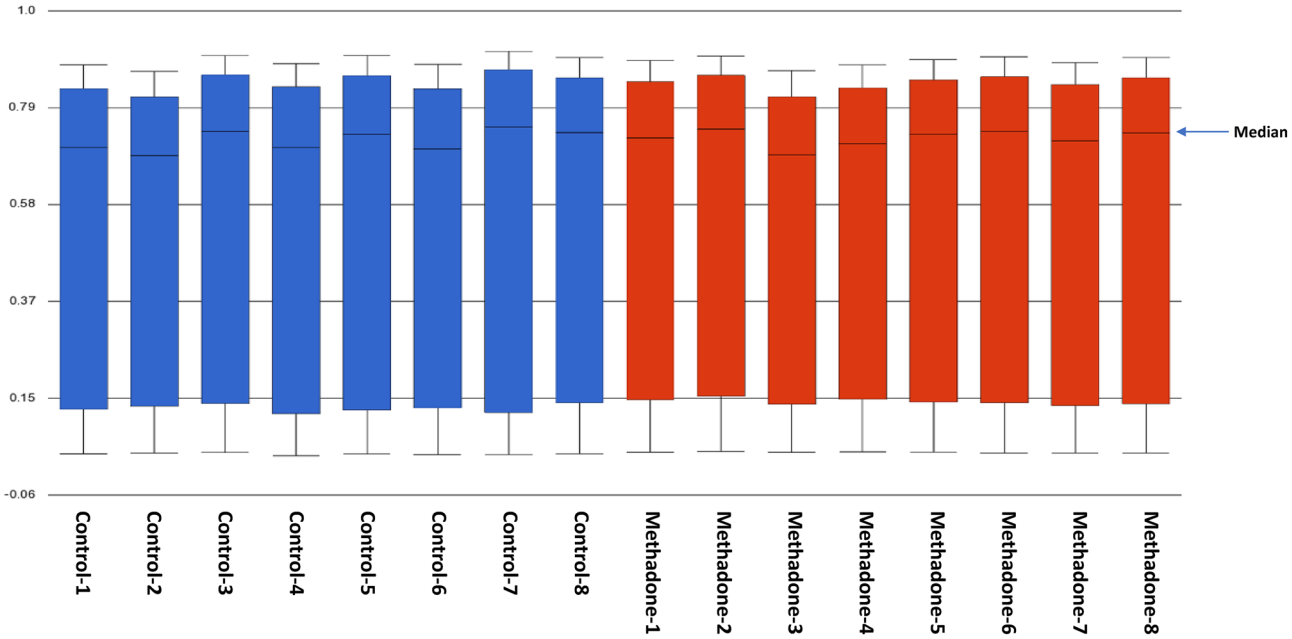

Figure 1. Box Plot showing β-distribution (methylation level) across all samples.

Column ID	Gene symbol	p-value	T-statistics	Beta difference (Methadone vs. control)
cg16551483	ZFP3	4.33E−05	5.83475	0.0174733
cg00356335	ANXA6	0.000569274	4.43149	0.480146
cg26703758	KCNC1	0.000699354	4.32469	0.59826
cg04066495	C2orf62	0.00165913	3.88212	0.411582
cg12259892	CPLX4	0.00280817	3.61596	0.36427
cg01081395	DNAJC6	0.00514763	3.31107	0.388559
cg21847720	MYOM2	0.00554426	3.27377	0.242467
cg14117320	PLEKHA7	0.00559642	3.26906	0.334204
cg18709904	C14orf182	0.00619057	3.21834	0.381908
cg13195461	YPEL1	0.00734786	3.13214	0.281014
cg11541881	C1orf21	0.00745155	3.12509	0.293026
cg17268094	ATP13A5	0.00860999	3.05232	0.315008
cg00905457	BLNK	0.00909557	3.02467	0.392127
cg26337497	OSBPL10	0.0122592	2.87385	0.311233
cg12657416	FAM69B	0.0123832	2.86875	0.386305
cg08063850	ATP9A	0.0136351	2.81991	0.293039
cg02507579	OR5H15	0.0138028	2.81371	0.415523
cg08292959	MGAT5B	0.0149818	2.77205	0.308533
cg12391372	SEMA4B	0.0162858	2.72955	0.359491
cg00167275	FAM35A	0.0177038	2.68693	0.0799136

Table 2. Top 20 hypermethylated CpGs associated with methadone exposure during pregnancy.

diseases and functions were modified with methadone exposure. Selected key diseases and functions altered with methadone exposure are listed in Table 5.

Tox functions

Methadone exposure during pregnancy potentially altered 26 tox functions in UCB (Table 6). Tox functions related to cardiotoxicity include cardiac failure, cardiac arrhythmia, cardiac dysfunction, cardiac enlargement, and congenital heart anomaly. Altered hepatotoxic functions are hepatocellular cancer, liver hyperproliferation, liver inflammation/hepatitis, liver cirrhosis, liver fibrosis, and liver cholestasis. Altered tox functions related to nephrotoxicity are renal failure, glomerular injury, renal nephritis, renal inflammation, and renal cell necrosis/cell death.

Column ID	Gene symbol	p-value	T-statistics	Beta difference (Methadone vs. control)
cg21838924	CLDN4	4.04E − 06	− 7.27852	− 0.549172
cg07304760	SND1	3.17E − 05	− 6.01537	− 0.41437
cg27413643	ANKRD27	0.000390315	− 4.62903	− 0.212522
cg22901347	TNIK	0.000769524	− 4.27526	− 0.510576
cg17330938	PPM1H	0.000886586	− 4.20229	− 0.348926
cg03643559	IKZF5	0.0047426	− 3.35226	− 0.275393
cg23854988	PHF21B	0.0050101	− 3.32468	− 0.452111
cg05845592	SULT1A1	0.00598517	− 3.2353	− 0.0920677
cg21463262	ATP11A	0.00651066	− 3.19299	− 0.33527
cg11986743	B4GALT6	0.00808566	− 3.08398	− 0.318985
cg15365500	UST	0.0104887	− 2.95275	− 0.36672
cg27494055	PCDHA10	0.0120504	− 2.88255	− 0.260356
cg15083522	LRRC27	0.0122725	− 2.8733	− 0.315761
cg04922606	FAM120B	0.0125607	− 2.86154	− 0.382339
cg04924408	SIAH3	0.0129081	− 2.84771	− 0.237718
cg19377607	LRRC20	0.0131091	− 2.83987	− 0.286636
cg10240906	BMP7	0.0142564	− 2.79728	− 0.34505
cg11251367	FMN2	0.0154978	− 2.75481	− 0.338259
cg08242313	DOCK10	0.0175354	− 2.69182	− 0.283316
cg06307915	CETP	0.0178121	− 2.68381	− 0.384047

Table 3. Top 20 hypomethylated genes associated with methadone exposure during pregnancy.

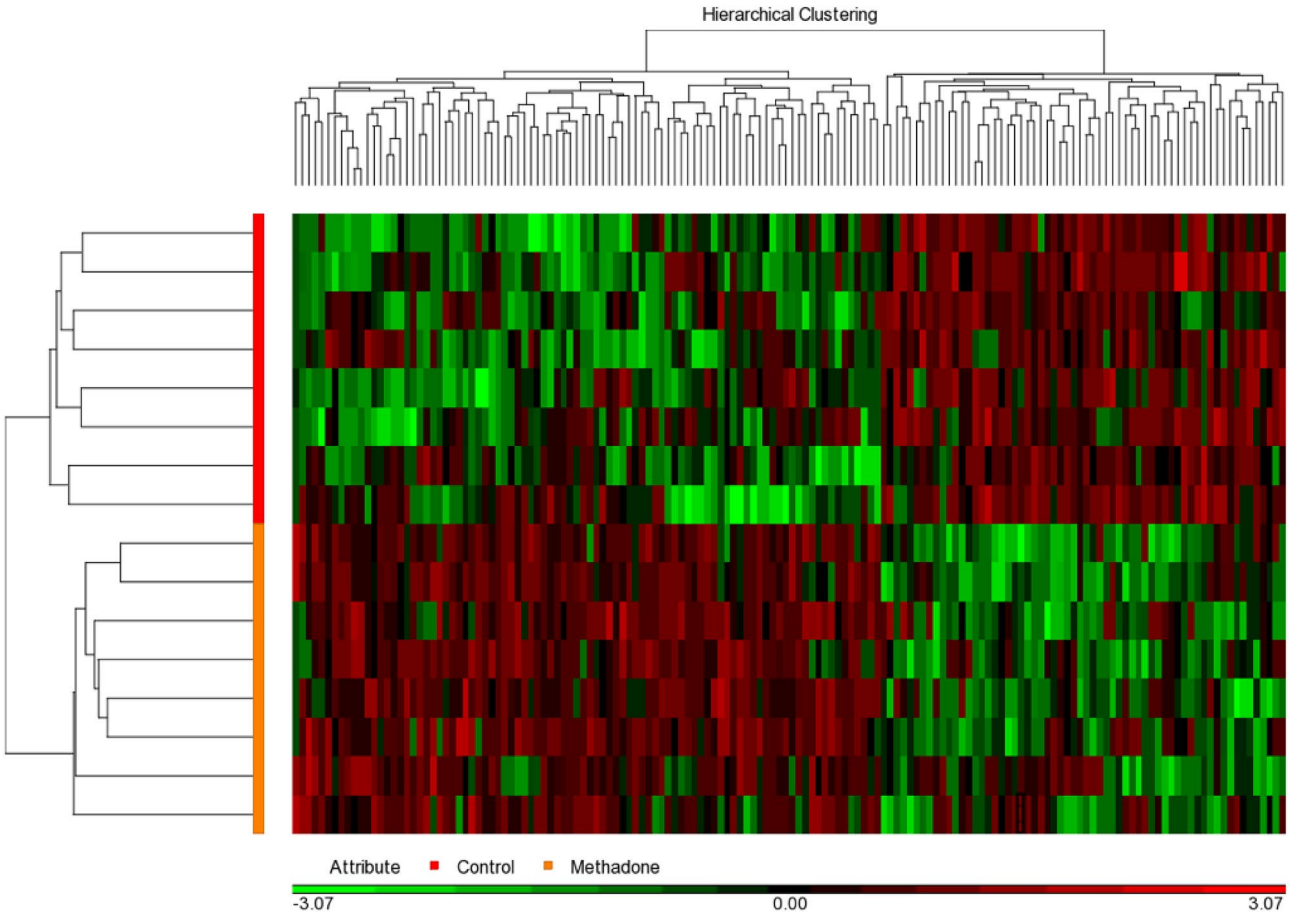

Figure 2. Cluster Analysis—Heatmap. From left to right are the 152 differentially methylated probe sets (FDR *P*-value ⩽ 0.50). From top to bottom are the control and methadone samples. The red and green colors indicate hyper-methylated and hypo-methylated loci, respectively.

Ingenuity canonical pathways	−log (p-value)	Molecules involved
LPS/IL-1 mediated inhibition of RXR function	2.55	CAT,CETP,MGST1,SULT1A1,UST
Xenobiotic metabolism signaling	2.17	CAT,MGST1,PIK3CG,SULT1A1,UST
Xenobiotic metabolism PXR signaling pathway	2.13	CAT,MGST1,SULT1A1,UST
NRF2-mediated oxidative stress response	1.86	CAT,DNAJC6,MGST1,PIK3CG
Th1 pathway	1.84	CD80,HLA-DQB2,PIK3CG
Th2 pathway	1.69	CD80,HLA-DQB2,PIK3CG
Chondroitin sulfate biosynthesis	1.65	SULT1A1,UST
Dermatan sulfate biosynthesis	1.62	SULT1A1,UST
Heparan sulfate biosynthesis	1.47	SULT1A1,UST
Superoxide radicals degradation	1.46	CAT
Purine ribonucleosides degradation to ribose-1-phosphate	1.46	PGM2L1
Role of JAK1 and JAK3 in γc cytokine signaling	1.46	BLNK,PIK3CG
Th1 and Th2 activation pathway	1.44	CD80,HLA-DQB2,PIK3CG
Growth hormone signaling	1.42	PIK3CG,SOCS7
Xenobiotic metabolism CAR signaling pathway	1.4	MGST1,SULT1A1,UST
Antiproliferative role of somatostatin receptor 2	1.38	GNG7,PIK3CG
Calcium transport I	1.37	ATP2B2
GDP-glucose biosynthesis	1.37	PGM2L1
Macrophage alternative activation signaling pathway	1.36	EPAS1,HLA-DQB2,PIK3CG
Glucose and glucose-1-phosphate degradation	1.33	PGM2L1
Glycogen degradation II	1.33	PGM2L1

Table 4. Canonical Pathways Picked Up by Ingenuity Pathway Analysis of the Differentially Methylated Genes in Infants of MMOD Mothers.

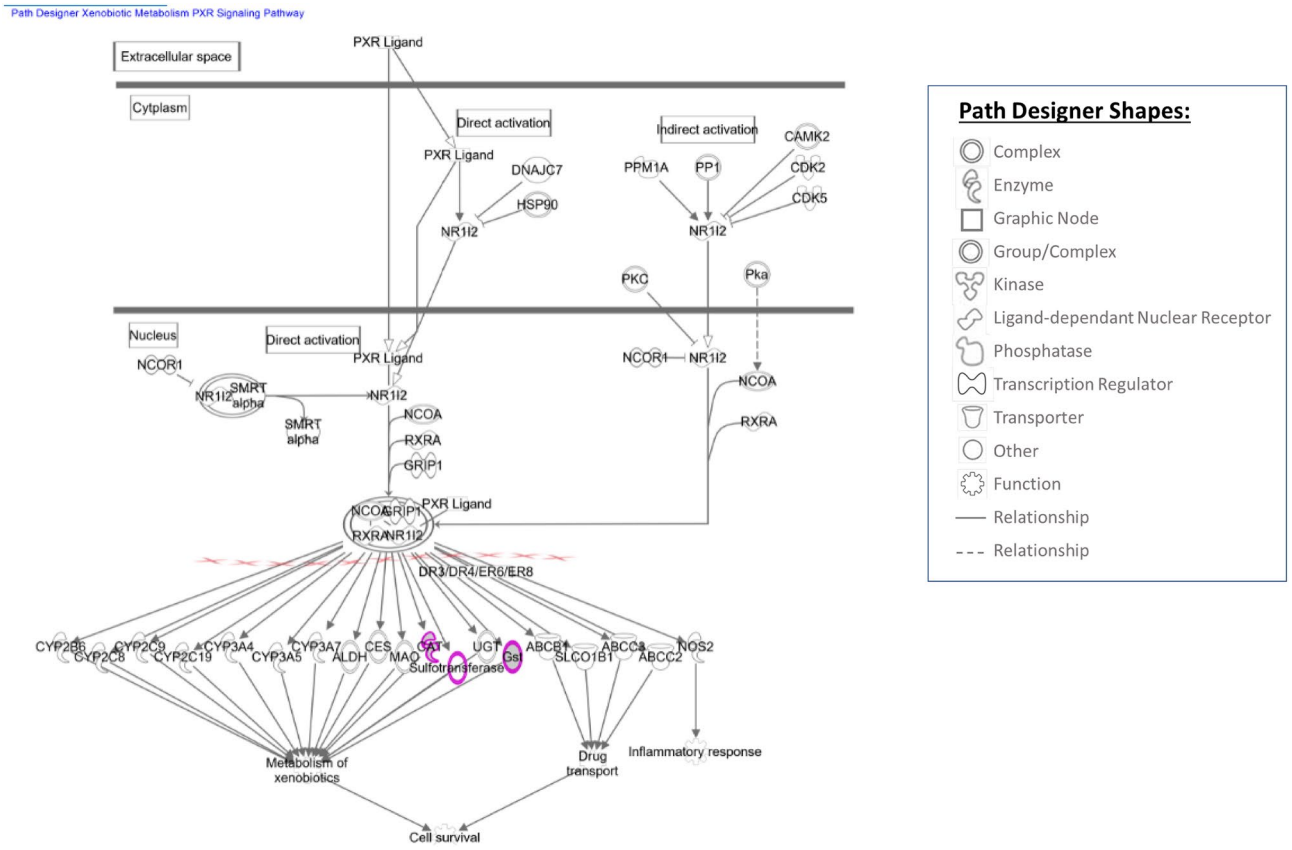

Figure 3. Xenobiotic Metabolism PXR Signaling Pathway—shows how xenobiotic metabolism could be varied in methadone exposure.

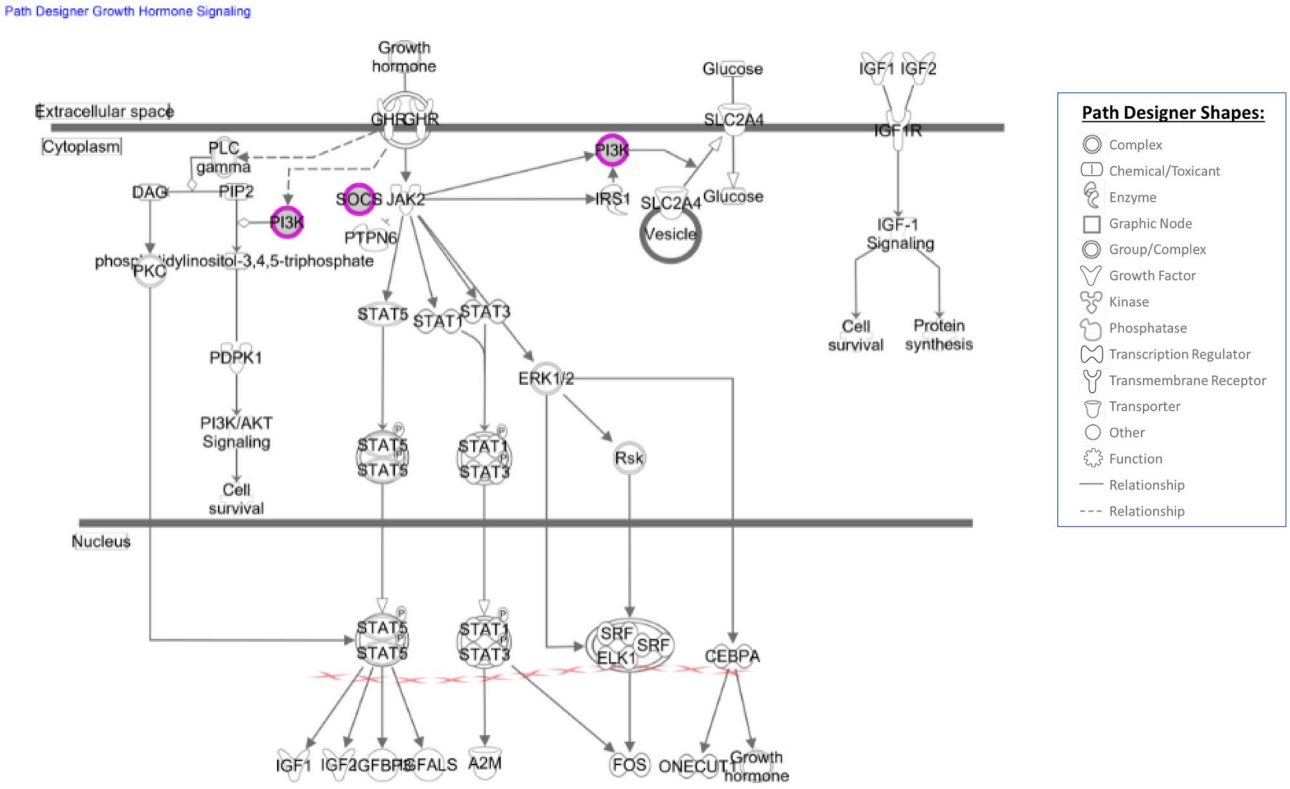

Figure 4. Growth Hormone Signaling Pathway—Depicts changes to growth hormone signaling detected by Ingenuity Pathway Analysis of the differentially methylated genes in umbilical cord blood of Infants exposed to maternal methadone.

Diseases and functions	Range of p-values for genes involved	Number of molecules involved
Gastrointestinal disease	$2.64E^{-05}$ to $4.98E^{-02}$	83
Organismal injury and abnormalities	$2.64E^{-05}$ to $4.98E^{-02}$	91
Neurological disease	$1.55E^{-04}$ to $4.9E^{-02}$	61
Cell death and survival	$2.79E^{-04}$ to $3.01E^{-02}$	7
Endocrine system disorders	$4.28E^{-04}$ to $4.98E^{-02}$	80
Molecular transport	$5.12E^{-04}$ to $4.53E^{-02}$	20
Hepatic system disease	$5.24E^{-04}$ to $2.82E^{-02}$	55
Respiratory disease	$6.71E^{-04}$ to $2.06E^{-02}$	46
Cardiovascular disease	$3.39E^{-03}$ to $4.27E^{-02}$	11
Auditory disease	$4.36E^{-03}$ to $3.01E^{-02}$	4
Behavior	$4.36E^{-03}$ to $4.24E^{-02}$	2
Cardiovascular system development and function	$4.36E^{-03}$ to $4.69E^{-02}$	2
Cellular growth and proliferation	$4.36E^{-03}$ to $3.65E^{-02}$	7
Developmental disorder	$4.36E^{-03}$ to $4.69E^{-02}$	17
Drug metabolism	$4.36E^{-03}$ to $1.3E^{-02}$	2
Free radical scavenging	$4.36E^{-03}$ to $4.9E^{-02}$	19
Ophthalmic disease	$4.36E^{-03}$ to $4.69E^{-02}$	15
Psychological disorders	$4.36E^{-03}$ to $3.43E^{-02}$	8

Table 5. Modified Diseases and Functions Obtained from Differentially Methylated Genes in Infants of MMOD Mothers.

Upstream regulators

Upstream regulator analysis by IPA identified 49 regulators to be dysregulated. Key identified regulators and their target molecules are described in Table 7.

Modified tox functions	Category	Range of p-values for genes involved	Molecules involved
Heart failure	Cardiotoxicity	$3.39E^{-03}$ to $4.01E^{-01}$	GRK5,SCN5A
Cardiac dilation		$4.36E^{-03}$ to $1.68E^{-01}$	NEDD4L,SCN5A
Cardiac enlargement		$4.36E^{-03}$ to $1.68E^{-01}$	NEDD4L,SCN5A
Tachycardia		$1.3E^{-02}$ to $1.27E^{-01}$	SCN5A
Congenital heart anomaly		$4.36E^{-03}$ to $9.11E^{-02}$	BMP7,MAML3,SCN5A
Cardiac dysfunction		$3.43E^{-02}$ to $3.43E^{-02}$	SCN5A
Cardiac arteriopathy		$3.51E^{-02}$ to $3.93E^{-02}$	ANKRD27,CETP,FMN2,LRRC20,SCN5A
Pulmonary hypertension		$3.26E^{-01}$ to $3.26E^{-01}$	SCN5A
Cardiac damage		$1.86E^{-01}$ to $1.86E^{-01}$	CAT
Cardiac inflammation		$9.16E^{-02}$ to $9.16E^{-02}$	CD80
Cardiac congestive cardiac failure		$4.01E^{-01}$ to $4.01E^{-01}$	SCN5A
Cardiac arrythmia		$4.36E^{-03}$ to $1.89E^{-01}$	PIK3CG,SCN5A
Liver hyperplasia/Hyperproliferation	Hepatotoxicity	$5.72E^{-04}$ to $1.6E^{-01}$	ACOT1,ADAMTSL1,ANKRD27,AQP12A/AQP12B, ARID1B,ATP11A,ATP13A5,ATP2B2,ATP9A, AUTS2,B4GALT6,BLNK,BMP7,C1orf21,CAT, CAVIN1,CDH10,CETP,CLDN4,DIPK1B,DOCK10, DYSF,FMN2,GZF1,HLA-DQB2,HPS5,LRRC27, MAML3,MED16,MGAT5B,MYOM2,MZF1, NDUFA10,OR5H15,PCDHA10,PIK3CG,PLEKHA7, PPM1H,SCN5A,SEMA4B,SHLD2,SIAH3,SLC38A9,SND1,SNTG2,SORBS2,SULT1A1,TANC1,TESPA1, TNIK,TRPS1,WRAP73
Liver cirrhosis		$3.96E^{-01}$ to $3.96E^{-01}$	CD80
Liver fibrosis		$3.96E^{-01}$ to $3.96E^{-01}$	CD80
Increased levels of alkaline phosphatase		$1.27E^{-01}$ to $1.27E^{-01}$	BMP7
Liver steatosis		$2.1E^{-01}$ to $2.37E^{-01}$	ACOT1,CAT
Liver inflammation/hepatitis		$2.37E^{-01}$ to $2.37E^{-01}$	ACOT1
Hepatocellular carcinoma		$8.56E^{-03}$ to $9.65E^{-02}$	ACOT1,ARID1B,ATP2B2,ATP9A,AUTS2,BMP7, CAT,CETP,DIPK1B,MAML3,OR5H15,PCDHA10, SND1,SULT1A1,TNIK
Liver cholestasis		$1.3E^{-02}$ to $2.82E^{-02}$	CAT,MGST1,SULT1A1
Renal damage	Nephrotoxicity	$5.93E^{-02}$ to $5.93E^{-02}$	CD80
Kidney failure		$9.51E^{-02}$ to $9.51E^{-02}$	CD80,SCN5A
Renal necrosis/Cell death		$1.16E^{-01}$ to $4.32E^{-01}$	ATP2B2,PAWR,SHLD2,TRPS1
Glomerular injury		$1.6E^{-01}$ to $1.6E^{-01}$	CD80
Renal inflammation		$1.6E^{-01}$ to $1.6E^{-01}$	CD80
Renal nephritis		$1.6E^{-01}$ to $1.6E^{-01}$	CD80

Table 6. Modified Tox Functions in Infants of MMOD Mothers.

Discussion

This pilot study found that there is differential DNA methylation in UCB cells from infants of MMOD persons involving multiple areas of body function. Exposure to methadone was associated with DNA methylation changes in genes that can contribute to neurological, neurobehavioral and growth abnormalities. Prior studies have observed increased risk of prematurity, smaller body measurements, increased susceptibility to opioid withdrawal and poorer neurodevelopmental outcomes with prenatal exposure to methadone[3–7]. In addition, Kelty, et al., showed association with increased risk of certain immune-related conditions like asthma and eczema in children with prenatal opioids exposure[17]. Although the mechanisms of these findings are not yet known, alteration in gene expression secondary to differential DNA methylation may potentially contribute to this increased risk.

The top hypermethylated genes observed with methadone exposure include *CPLX4* (complexin 4), ZFP3 (ZFP3 Zinc Finger Protein), *PLEKHA7* (pleckstrin homology domain containing A7), *KCNC1* (potassium voltage-gated channel subfamily C member 1), *TANC1* (Tetratricopeptide Repeat, Ankyrin Repeat and Coiled-Coil Containing 1) and *AUTS2* (Activator of Transcription and Developmental Regulator AUTS2). *CPLX4* is associated with encoded protein involved in synaptic vesicle exocytosis. It has been linked to X- linked cone rod dystrophy which manifests as reduced visual acuity and sensitivity in the central visual field, leading to eventual peripheral vision loss and severe impairment of overall vision[18]. As mentioned above, some studies have associated prenatal methadone exposure to visual abnormalities that has not yet been well explained. Identifying the effect of hypermethylated CPLX4 on gene expression will be an important next step. ZFP3 is a protein coding gene that is suggested to enable DNA-binding transcription factor activity, RNA polymerase II-specific and RNA polymerase II transcription regulatory region sequence-specific DNA binding activity[19]. *PLEKHA7*, enables delta-catenin binding activity which is involved in epithelial cell–cell adhesion; pore complex assembly; and zonula adherens maintenance. It is found in several cellular components, including the centrosome; nucleoplasm; and zonula adherens. Diseases associated with *PLEKHA7* include cleft lip with or without cleft palate and primary angle-closure glaucoma[20]. *KCNC1* (potassium voltage-gated channel subfamily C member 1), plays an important role in the rapid repolarization of fast-firing brain neurons. Among its related pathways are potassium channels and transmission across chemical synapses[21]. *TANC1* (Tetratricopeptide Repeat, Ankyrin Repeat and Coiled-Coil

Upstream regulators	−log (p-value)	Molecule type	Molecules involved
CYP2E1	0.000113	Enzyme	CAT,MGST1
mir-30	0.000667	microRNA	CAT,PLEKHA7
OTUD6B	0.00437	Peptidase	EPAS1
ACOT8	0.00437	Enzyme	CAT
PRB1/PRB2	0.00437	Other	SERPINB2
RARRES1	0.00437	Other	GRK5
CDH3	0.00618	Other	BMP7,PLEKHA7
H2BC17	0.00809	Other	PIK3CG,TNIK
P4HTM	0.00872	Enzyme	EPAS1
ABCB6	0.00872	Transporter	CAT
HTR2B	0.00872	G-protein coupled receptor	SERPINB2
USP33	0.00872	Peptidase	EPAS1
NOX1	0.00872	Enzyme	EPAS1
TCF4	0.0123	Transcription regulator	BMP7,MRTO4,PIK3CG,SORBS2
ARF4	0.0131	Enzyme	CLDN4
EGLN2	0.0131	Enzyme	EPAS1
DDX28	0.0131	Enzyme	EPAS1
LPA	0.0131	Other	SERPINB2
HOXC11	0.0131	Transcription regulator	PAWR
ADORA2	0.0174	Group	CD80
EHD2	0.0174	Other	CAVIN1

Table 7. Key Upstream Regulators Affected by Exposure to Methadone During Pregnancy.

Containing 1) is predicted to be involved in the regulation of post synapse organization. *TANC1* is associated with intellectual developmental disorders[22]. Likewise, *AUTS2* (Activator of Transcription and Developmental Regulator AUTS2) has been implicated in neurodevelopment and identified as a candidate gene for numerous neurological disorders, including autism spectrum disorders, intellectual disability, and developmental delay[23]. Notably, neurodevelopmental delay has been reported in infants of MMOD mothers. Investigating alterations in the expression of these genes affected by DNA methylation and establishing correlations with neurodevelopmental outcomes represents a crucial next step in understanding the observed association. Top Hypomethylated genes include *CLDN4* (claudin 4), *TNIK* (TRAF2 and NCK Interacting Kinase), *ATP11A* (ATPase Phospholipid Transporting 11A*), BMP7* (Bone Morphogenetic Protein 7), *FMN2* (Formin 2), and *SCN5A* (Sodium Voltage-Gated Channel Alpha Subunit 5). *CLDN4* functions as a crucial membrane protein in the composition of epithelial cell tight junctions, regulating movement of solutes and ions through the paracellular space. *CLDN4* is also believed to play a possible role in internal organ development and function during pre- and postnatal life. Its absence has been associated with Williams-Beuren syndrome, a neurodevelopmental disorder affecting multiple systems[24]. *TNIK* expression plays important roles in carcinogenesis and embryonic development. A mutation in this gene is associated with intellectual developmental disorder, characterized by significantly below-average general intellectual functioning accompanied by impairments in adaptive behavior, typically observed during the developmental period. Individuals with this disorder often exhibit intellectual disability, delayed speech, and hyperactivity[25]. *ATP11A,* which is integral to membrane ATPase function, has been linked with leukodystrophy, hypomyelinating diseases and deafness[26]. Abnormal myelination has been reported to infants of MMOD mothers[27]. *BMP7* codes a ligand of growth factor of the TGF-beta superfamily that plays important role in various biological processes, including embryogenesis, hematopoiesis, neurogenesis, and skeletal morphogenesis[28]. Diseases associated with *BMP7* include multiple types of congenital heart defects[29]. *FMN2* encoded protein is thought to have essential roles in organization of the actin cytoskeleton and in cell polarity. Mutations in this gene have been associated with infertility and with an autosomal recessive form of intellectual disability[30].

SCN5A protein mediates the voltage-dependent sodium ion permeability of excitable membranes. It forms a sodium-selective channel through which Na (+) ions may pass in accordance with their electrochemical gradient. Over expression may impair the function of excitable membranes[31]. Diseases associated with *SCN5A* include sudden infant death syndrome and long QT syndrome. Methadone use in adults can lead to prolongation of QTc through inhibition of the cardiac ion channel KCNH2 in a dose dependent manner[32]. In infants of MMOD mothers, there have also been reports of QTc prolongation in the first 2 days of life with subsequent normalization[33]. The mechanism of neonatal QTc prolongation related to MMOD has not been described. While we may assume it mirrors what was described in adults, it may also be related to epigenetic changes in certain excitable membranes like the observations made in the *SCN5A* gene.

Altered methylation pattern in genes involved in canonical pathways can potentially link methadone exposure during pregnancy to short- and long-term outcomes in offspring. At least 325 canonical pathways were found to be altered in UCB cells exposed to methadone during pregnancy, in this study. *OPRM1*, a mu-opioid receptor related gene, has been shown to be differentially methylated with methadone exposure and may play

a role in NOWS risk or substance dependence later in life[34]. While this pilot study did not observe increased methylation with the *OPRM1* gene, our data indicate that xenobiotic metabolism PXR signaling pathway and xenobiotic metabolism CAR signaling pathway were altered in infants of MMOD mothers. This change could imply an adaptive response to opioid exposure in the same manner as of increased methylation of *OPRM1* with resultant varied response to opioid exposure later in life.

Moreover, we found that intrauterine exposure to methadone was associated with increased DNA methylation in genes related to the NRF2-mediated oxidative stress response pathway and superoxide radicals' degradation pathways. Leventelis C, et al. and another study reported significant oxidative stress response with associated evidence of compromised antioxidant defense in adults exposed to heroin who were maintained on methadone[35,36]. Altered oxidative stress response in infants of MMOD may be reflective of in utero adaptation to methadone exposure. Oxidative stress has been shown to be detrimental to neuronal development. Obst S, et al. found that increased reactive oxidative species (ROS) have detrimental effects on oligodendrocyte maturation, myelination, and neuronal survival, leading to ultrastructural abnormalities of myelin formation and grey matter injury[37]. Abnormal oligodendrocyte maturation and altered maturation of connective tracts have been associated with infants of MMOD mothers with suggestion that this finding may be the basis of the increased risk for cognitive and behavioral difficulties observed in children of mothers using opioids.[27,38].

Altered intracellular growth hormone (GH) signaling pathways involving PIK3CG and SOCS7 molecules can affect the synthesis of insulin-like growth factors (IGF) associated with increased risk of fetal growth restriction observed in infants of MMOD mothers. Intrauterine human growth requires the normal expression of IGF-I/IGF-II and type 1-IGF receptor[39]. While IGF-receptor genes have not been implicated in the canonical pathway, IGF-I and IGF-II expression can be impaired secondary to upstream cytoplasmic alteration in growth hormone signaling. Fetal growth in the third trimester is mostly influenced by nutritional and other maternal factors and less by fetal genetics. Nonetheless, persistent abnormalities in the GH-IGF axis have been implicated in small for gestational age infants and fetal growth restriction[40].

Exposure to methadone during pregnancy was associated with alterations in 26 toxic functions in our cohort. The top toxic functions that altered DNA methylation were seen in genes related to hepatoxicity, cardiotoxicity, and nephrotoxicity. The mechanism of cardiac rhythm abnormalities in infants of MMOD is not well described and epigenetic factors may play a contributory role.

To our knowledge, this is the first study reporting genome wide differential DNA methylation in UCB cells from infants exposed to methadone during pregnancy. The altered methylation pattern identified in genes relate to neurodevelopmental delay, neurobehavioral disorders, oxidative stress and growth function show a potential connection to the reduced neurodevelopmental and anthropometric measures observed in infants of MMOD mothers. An important follow up study is investigating how differential methylation influences gene expression in these cohorts of infants. Further studies, preferably with a larger cohort, is needed to investigate a cumulative dose-dependent effect of methadone on DNA methylation patterns and if observed changes in DNA methylation persist through out the neonatal period and if it correlates with certain outcomes seen in infants of MMOD mothers.

Our study has its limitations, particularly, the small sample size of 16 neonates, but similar limited sample sizes have been used in studies investigating differential DNA methylation patterns[41,42]. There is a chance of finding differences in DNA methylation due to multiple comparisons which was controlled by employing a FDR p-value < 0.05, and a beta difference of $> +2$ or < -2. Our results should be considered as hypothesis generating and should be validated in a larger cohort. Also, other maternal exposures including other substances used and maternal psychosocial stressors during pregnancy may contribute to the methylation changes observed.

In conclusion, methadone exposure during pregnancy is associated with differential DNA methylation in UCB cells. We identified 97 differentially methylated genes, important tox functions, upstream regulators and canonical pathways related to the oxidative stress, xenobiotic metabolic response, and cardiotoxicity in UCB cells of infants of MMOD mothers. Future studies can further validate differential methylation of target genes in a larger cohort of infants. Our data contribute to a deeper understanding of the impact of methadone exposure during pregnancy on both short-term and long-term outcomes, highlighting the significance of key pathways and genes. Functional studies on these identified pathways and genes could enhance our understanding of the underlying mechanisms, ultimately guiding the development of effective interventions.

Methods

Ethical, human study protocol and institutional biosafety approvals

All human protocols and procedures described in this study were approved by the Institutional Review Board of Thomas Jefferson University Hospital. All experiments performed in this study were approved by the Nemours Institutional Biosafety Committee. All methods were performed in accordance with the relevant guidelines and regulations. The Institutional Review Board (IRB) waived informed consent as the study was performed on discarded blood and placental tissue samples.

Study design

This is a pilot prospective observational study to examine differential DNA methylation in UCB cells of term and near-term neonates born to MMOD mothers. Samples of UCB and placental tissue were collected at the time of delivery from full term and near term (≥ 35 weeks) infants. Exclusion criteria included infants with fetal growth restriction, clinical or histological chorioamnionitis and major congenital/chromosomal anomalies.

UCB collection

The UCB was obtained on the delivery table immediately after separation of baby from placenta. When this method was not feasible, a long segment of the cord was obtained and cleaned with 70% alcohol prior to collection of samples using a needle and syringe or a butterfly needle. The UCB was collected in PAXGene blood DNA tube (BD Catalog # 7611650), processed on the day of collection as per manufacturer's protocol and saved at – 80 °C.

Fetal membrane collection, processing, staining, and diagnosis of hierarchical cluster analysis (HCA)

The placental tissue was processed and kept in 10% neutral buffered formalin (NBF) for 24–48 h before it was transferred to 70% alcohol. Tissue samples were processed using standard operating procedures at the histopathology laboratory at Thomas Jefferson University and paraffin embedded in Histoplast LP (Thermo Fisher Scientific, Fremont, CA). The samples were then classified as having HCA or no HCA by a blinded pathologist (JC). We collected information on HCA as it is a potential confounder in assessing for DNA methylation based on a prior study showing that epigenetic changes occur via DNA methylation in infants exposed to HCA[43,44].

DNA isolation and statistical approaches for DNA methylation analysis

DNA isolation was performed using QIAamp DNA Mini kit (Qiagen, Germantown, MD). DNA was quantified on a Qubit 2.0 fluorometer, (Thermo Fisher Scientific, Waltham, MA), and the DNA quality was assessed by an Agilent 2200 TapeStation (Agilent Technologies, Palo Alto, CA). The genome-wide DNA methylation study was performed using the Illumina Methylation EPIC Array (cat# WG-317-1001, Illumina Inc., San Diego, CA). Illumina iScan Reader was used to analyze the image and data from Methylation EPIC Bead Chip. Data processing was performed with Illumina GenomeStudio software. Raw IDAT files were processed using Partek Genomics Suite V.7.20 (Partek Inc. Missouri, USA) and annotated using the MethylationEPIC_v-1-0_B4 manifest file. Probes from the X and Y chromosomes were excluded from the study (since we are having both males and females in the samples), and probes based on detection with $P > 0.05$ were also filtered to exclude low-quality probes. Background normalization was performed using Swan Normalization. Principal component analysis (PCA) was performed to visualize clusters in the methylation data, and as a quality control procedure (Fig. 5). Distribution of β-values across the samples was inspected by a box-and-whiskers plot (Fig. 1). Samples were attributed to the two groups, methadone exposed and control. Differential methylation analysis was then performed between the two groups at a p-value < 0.05, and a difference of $> +2$ or < -2. To detect the differential methylation in global CpGs that varies across all samples, we performed a 1-way ANOVA test. Hierarchical cluster analysis of the significant CpGs was carried out with the Heatmap function in the Partek Genomics Suite (Fig. 2). Clinical data were compared with Fisher's exact test and Wilcoxon rank-sum test using Stata Statistical Software 15 College Station, TX: Stata Corp LLC.

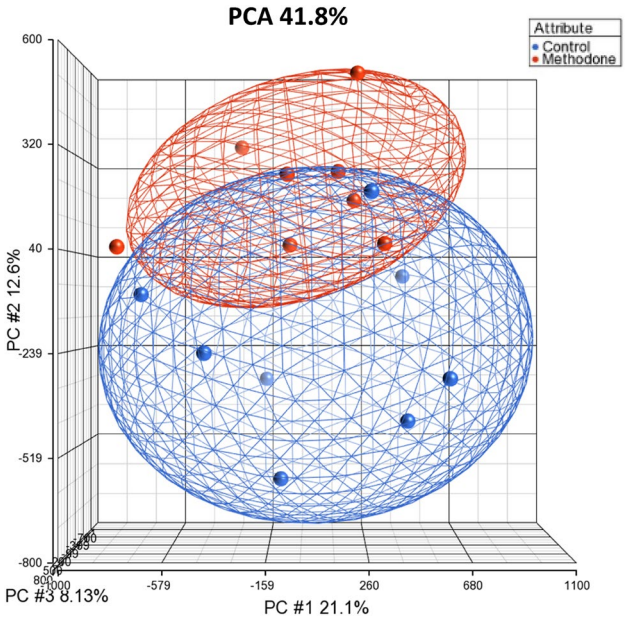

Figure 5. Principal components analysis (PCA) in 3D showing methylation profiles of the study samples. Each sample is represented by a dot, the axes are the first three Principal Components (PCs), the percentages indicate the fraction of variance explained by each PC.

Data availability

The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request. Data are in an encrypted server with Thomas Jefferson University.

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Author contributions

O.A: Sample collection, processing, data analysis, manuscript write up and editing. SGB: Sample collection and processing, data interpretation, manuscript write up and editing. PU: Data collection and manuscript writing. RH: Data collection and manuscript writing. KB: Data collection and manuscript writing. HBA: Concept and design, data interpretation manuscript writing. KS: Concept and design, data interpretation, manuscript writing. JSC: Concept and design, data interpretation, placental tissue analysis, manuscript writing. SA: Concept and design, data collection, sample processing, data analysis and interpretation, and manuscript writing. RCB: concept and design, data interpretation, manuscript writing. Z.A: Concept and design, data analysis and interpretation, manuscript writing.

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Competing interests

The authors declare no competing interests.

Additional information

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