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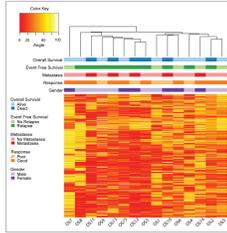
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Novel DNA methylation profiles associated with key gene regulation and transcription pathways in blood and placenta of growth-restricted neonates

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Novel DNA methylation profiles associated with key gene regulation and transcription pathways in blood and placenta of growth-restricted neonates

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Keywords: DNA methylation, fetal growth restriction, fetal origins of adult disease, Marmal-aid, placenta, transcription factor, umbilical cord

Abbreviations: AGA, appropriately grown offspring; BMI, body mass index; CG, cytosine phosphate guanine loci; DMP, differentially methylated positions; FDR, false discovery rate; FGR, fetal growth restriction; GO, gene ontology; HOMA, homeostasis model assessment; ICR1, imprinting control region 1; MODY, maturity onset diabetes of the young; T2DM, type 2 diabetes mellitus; UCL, University College London; UCLH, University College London Hospital; UCSC, University of California Santa Cruz

Fetal growth is determined by the fetoplacental genome interacting with the maternal in utero environment. Failure of this interplay leads to poor placental development and fetal growth restriction (FGR), which is associated with future metabolic disease. We investigated whether whole genome methylation differences existed in umbilical cord blood and placenta, between gestational-matched, FGR, and appropriately grown (AGA) neonates. Using the Infinium HumanMethylation450 BeadChip[®], we found that DNA from umbilical cord blood of FGR born at term ($n = 19$) had 839 differentially methylated positions (DMPs) that reached genome-wide significance compared with AGA ($n = 18$). Using gestational age as a continuous variable, we identified 76,249 DMPs in cord blood (adj. $P < 0.05$) of which 121 DMPs were common to the 839 DMPs and were still evident when comparing 12 FGR with 12 AGA [39.9 ± 1.2 vs. 40.0 ± 1.0 weeks (mean \pm SD), respectively]. A total of 53 DMPs had a β methylation difference $>10\%$ and 25 genes were co-methylated more than twice within 1000 base pairs. Gene Ontology (GO) analysis of DMPs supported their involvement in gene regulation and transcription pathways related to organ development and metabolic function. A similar profile of DMPs was found across different cell types in the cord blood. At term, no DMPs between FGR and AGA placentae reached genome-wide significance, validated with an external dataset. GO analysis of 284 pre-term, placental DMPs associated with autophagy, oxidative stress and hormonal responses. Growth restricted neonates have distinct DNA methylation profiles in pre-term placenta and in cord blood at birth, which may predispose to future adult disease.

Introduction

Fetal growth restriction, defined as an estimated fetal weight <10 th customized centile for gestational age and adjusted for maternal weight, height, parity, and ethnicity, is a major cause of neonatal morbidity and mortality, associated with childhood neurocognitive delay and metabolic and cardiovascular disease in later life.^{1–3} To fulfill its growth potential, a fetus must assimilate

an adequate nutritional supply from its mother across the placenta. This process is governed by a complex interplay between the fetoplacental genome and the maternal environment.⁴ In low-income nations, environmental influences on fetal growth are predominantly related to poor maternal nutrition, chronic disease or infections.⁵ In high-income nations, fetal growth is more commonly compromised by poor placental development.⁶

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Poor placental development affects approximately 4–7% of all first time pregnancies and is often associated with the maternal gestational syndrome preeclampsia, which is characterized by maternal hypertension and proteinuria.⁷ Inadequate placental invasion may be due to adverse immune events at the maternal-placental interface and impaired oxygen sensing by the placenta.^{8,9} In these circumstances, high resistance to blood flow in utero-placental arteries pre-dates the onset of FGR.⁶ Inadequate placental development might then lead to epigenetic change in the under nourished fetus. An example of this sequence of events has been observed in a rodent model when uterine artery blood flow was artificially reduced during pregnancy.¹⁰ As a consequence, the growth restricted offspring developed diabetes in adulthood, associated with reduced pancreatic β cell mass.¹⁰ These events were associated with DNA methylation and reduced expression of *PDX1*, a transcription factor critical to pancreatic cell development.¹⁰

A genome wide approach has been taken to investigate methylation differences between 5 FGR and 5 AGA offspring, using hematopoietic CD34+ stem cells isolated from umbilical cord blood.¹¹ In FGR offspring, there was a low level of methylation difference (6%) in a promoter of *HNF4A*, a gene associated with maturity onset diabetes of the young (MODY). Altered methylation status of selected candidate genes in umbilical cord tissue has also been correlated with later life phenotype.¹² However, these findings have not been reproduced in prospectively collected umbilical cord blood, or placental samples from babies born with growth restriction, or across a range of birth weights investigated in later life.^{13–15} Furthermore, gestational age at delivery has a powerful effect on fetal and placental methylation status,^{16,17} which has not always been considered in earlier studies of FGR offspring.

Genetic factors inherited from either parent also influence placental development and fetal growth.^{18,19} Gene variants associated with insulin resistance are associated with reduced fetal growth and provide a potential genetic link between low birth weight and future risk of type-2 diabetes.²⁰ Other genes have conflicting influences on placental development and fetal growth according to their parent of origin. Genomic imprinting describes monoallelic expression of a gene following epigenetic silencing of the other allele.²¹ For example, hypomethylation of CpG sites on imprinting control region 1 (ICR1) of 11p15 reduces expression of *IGF2* and leads to reduced fetal growth,

whereas hypermethylation of ICR1 increases expression of *IGF2* and is associated with fetal overgrowth.²² Fetal growth restriction secondary to known imprinted gene disorders are however rare and do not explain the majority of FGR seen in the developed world.

In order to discover novel DNA methylation differences associated with FGR in the absence of maternal and fetal disease and in gestational age-matched subjects, we conducted a case-control study that identified otherwise healthy pregnant women with either FGR (cases) or appropriately grown offspring (AGA; controls). We used the Infinium HumanMethylation450 BeadChip[®] to discover differences in the whole methylome (450,000 CpG sites in 99% of genes) of umbilical cord blood and placentae from cases and controls. After controlling for gestational age, we identified the most differentially methylated positions between FGR and AGA offspring and recognized that DMPs were consistent across different cord blood cell types. Gene ontology (GO) pathway analysis associated these DMPs with gene regulation and transcription pathways related to organ development and metabolic function. These novel DNA methylation profiles provide a robust platform for further investigation into their role in the fetal origins of adult diseases.

Results

Participant phenotype

Fetal growth restricted offspring identified in utero were confirmed at birth (FGR; $1.9\% \pm 2.3\%$, customized birth weight centile) and compared with appropriately grown for gestational age offspring (AGA; $51.8\% \pm 30.5\%$, customized centile). Retrospective review of fetal ultrasound scans at 20–22 weeks gestation showed that 90% (26/29) of mothers later identified with FGR offspring had high resistance to utero placental blood flow. No abnormal recording of utero-placental blood flow was made at 20–22 weeks in pregnancies that went on to have AGA offspring.

Mothers of both cases and controls met the study inclusion criteria and were of similar age, body mass index (BMI) and parity, and did not smoke (Table 1; see Materials and Methods).

Fathers of FGR offspring were more insulin resistant, as judged by fasting HOMA IR, and had larger waist circumferences compared with fathers of AGA offspring (Table 1).

Table 1a. Baseline characteristics of the parents according to offspring birth weight. (AGA, appropriately grown for gestational age, or FGR, fetal growth restriction).

	AGA mothers (mean \pm SD)	AGA fathers (mean \pm SD)	FGR mothers (mean \pm SD)	FGR fathers (mean \pm SD)
Number of parents	24	24	29	29
Age (years)	31.9 \pm 3.98	33.8 \pm 4.7	32.8 \pm 2.98	34.8 \pm 5.3
BMI (kgm ⁻²)	23.7 \pm 3.5	25.7 \pm 3.2	23.4 \pm 3.6	27.1 \pm 2.8
Smoker	0	6 (25%)	0	9 (31%)
Abdominal circumference (cm)		92.1 \pm 7.7		94.8 \pm 7.1
Insulin resistance (HOMA index)		0.6 \pm 0.22		1.1 \pm 0.46
Maternal parity	Primigravid, n = 21 (77%)		Primigravid, n = 25 (86%)	

Table 1b. Pregnancy characteristics according to offspring birth weight (FGR or AGA), nature of samples analyzed (cord blood or placenta), and gestational age at birth (all samples, or term > 255 d gestation). Results expressed as mean \pm SD

	All AGA offspring (mean \pm SD)	All FGR offspring (mean \pm SD)	Term FGR offspring (mean \pm SD)
CORD BLOOD			
Number of samples	18	27	19
Birth weight (grams)	3634 \pm 369	2065 \pm 770	2487 \pm 420
Customized centile (%)	51.8 \pm 30.5	1.9 \pm 2.3	2.6 \pm 2.3
Gestation (range) days	285 (272–299)	258 (202–293)	272 (256–293)
Baby sex	Male, n = 9 Female, n = 9	Male, n = 9 Female, n = 18	Male n = 7 Female n = 12
PLACENTA			
Number of samples	23	22	15
Birth weight (grams)	3614 \pm 361	2011 \pm 710	2428 \pm 342
Customized centile (%)	47.6 \pm 28.6	1.8 \pm 2.3	2.3 \pm 2.0
Gestation (range) days	285 (266–300)	257 (202–286)	272 (256–286)
Baby sex	Male, n = 11 Female, n = 12	Male, n = 9 Female, n = 13	Male, n = 6 Female, n = 9
Mode of delivery	Vaginal, n = 18 (75%)		Vaginal, n = 12 (66.7%)
(term-pregnancies only)	Caesarean section, n = 6		Caesarean section, n = 6

Differentially methylated positions in cord blood and placenta from growth restricted offspring compared with appropriately grown offspring

Initial analysis included 45 umbilical cord blood samples (FGR n = 27 and AGA n = 18) and 45 placental samples (FGR n = 23 and AGA n = 22). Differentially methylated positions (DMPs) were identified using t test-based statistics in the Limma package provided by Bioconductor 2.11.²³ Adjustment for genome-wide significance was performed using a False Discovery Rate (FDR) with a threshold p value for DMPs of <0.05.²⁴ Irrespective of gestational age, there were 553 differently methylated positions in cord blood and 453 DMPs in placenta that reached genome wide significance between all FGR and AGA offspring (adj. $P < 0.05$). As all AGA offspring were born at term, but only 19/27 FGR offspring, it was possible that these DMPs were influenced by gestational age.

Effect of gestational age on umbilical cord blood DMPs between FGR and AGA offspring

We compared genome-wide methylation differences between FGR cases (n = 19) with AGA controls (n = 18) that only delivered >255 d (36.4 weeks). This analysis yielded 839 DMPs in the cord blood between FGR and AGA offspring, of which the majority (86%) were hypermethylated (Fig. 1). From the original 553 cord blood DMPs, 371 DMPs (67%) were also common to the 839 term DMPs and may therefore be secondary to fetal growth restriction, rather than the effect of gestational age.

To further elucidate the effect of gestational age on DMPs between FGR and AGA cord blood, we ran a linear model on all cord blood samples (FGR and AGA) using gestational age as a continuous

variable. Across the gestational age range (202 d to 299 days) there were 76,249 DMPs in cord blood (adjusted $P < 0.05$). The majority of DMPs were characterized by hypomethylation (68%), an assessment that was heavily influenced by samples from pre-term FGR offspring.

We then compared the 839 DMPs evident between FGR and AGA offspring at term with the 76,249 DMPs found in the gestational age cord blood model and identified 121 DMPs that were common to both. We compared the direction of methylation of these 121 DMPs from both groups and found that in all cases their direction of methylation was identical. Furthermore, the same 121 DMPs were still evident when comparing 12 FGR and 12 AGA offspring that were more exactly matched for gestational age (279.1 \pm 8.3 vs. 280.3 \pm 7.2 days, respectively).

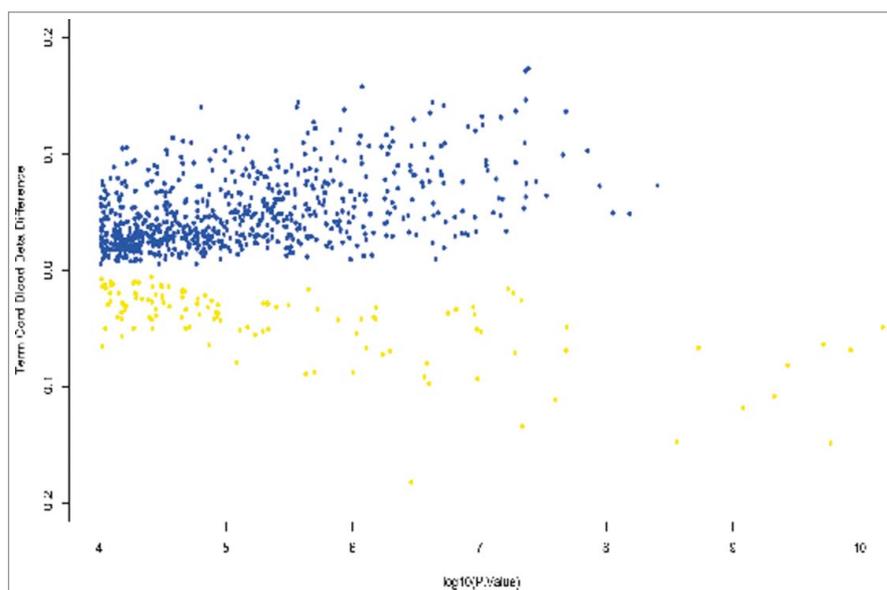


Figure 1. Volcano plot showing differentially methylated positions in umbilical cord blood between growth restricted (FGR) and appropriately grown offspring (AGA) at term gestation. β values are plotted against log P values. Hypermethylated positions are shown in blue and hypomethylated positions in yellow. A β methylation value of 10% is represented as 0.1.

We also compared our results to another study of a separate cohort of AGA offspring that investigated DMPs within whole cord blood between 32–43 weeks gestation.¹⁶ Using the Human-Methylation27 BeadChip, 25 DMPs were identified in whole umbilical cord blood, related to gestational age. None of these 25 gestational age-related DMPs were present in our 121 FGR cord blood DMPs at term. This observation does not diminish the possibility that the 121 DMPs relate to fetal growth rather than gestational age.

Effect of cell type on umbilical cord blood DMPs between FGR and AGA offspring

We investigated whether the 839 DMPs between FGR and AGA might be due to differences in cellular composition of umbilical cord blood. We downloaded 450K data from the publicly available database Marmal-aid,²⁵ which incorporates data from multiple studies that has been normalized and quality controlled. Data was available on circulating cell subtypes identified as CD4, CD14, CD8, CD19, CD56, peripheral blood leucocytes, whole blood or peripheral blood mononuclear cells from 109 different samples. We plotted the 839 different probes for the 109 different samples and grouped the samples into their cellular subtypes (Fig. 2). Overall, there was very little difference in methylation between the 839 DMPs according to cell subtype. This is portrayed as similar color profiles for each of the cell types (blue, yellow, and green) that correspond to the level of β methylation (far column) of the 839 probes (Fig. 2).

In order to identify whether there was a pattern of methylation within a single cell subtype, we plotted the 839 DMPs

between FGR and AGA in cord blood (second column, Fig. 2). There was no such correlation with a single cell subtype, which supports the conclusion that DMPs between FGR and AGA in cord blood are unlikely to be due to different cell proportions.

Leucocyte number is increased in neonates compared with adults, but the absolute number of CD4 and CD19 lymphocytes or dendritic cells is not different between pre-term or term neonates.²⁶ The absolute CD14 monocyte count is lower in very pre-term neonates compared with late pre-term and term neonates, but the relative frequency of CD14 monocyte cells is not different, at around 3.5% of all leucocytes.²⁶

Identity of most differentially methylated positions between FGR and AGA offspring in umbilical cord blood

We stratified umbilical cord blood DMPs between FGR and AGA offspring born at term, according to their degree of methylation difference. Of the 839 DMPs, 304 had a β methylation difference of >5% and 53 DMPs had a β methylation value difference of >10% (Table 2a). The majority of DMPs with a β methylation >10% were hypermethylated (41/53, 77%; Table 2a) and 8/53 were hypomethylated >-10% (Table 2b). Of the 839 DMPs that were identified on Ref-seq annotated genes, 658 DMPs were within 1000 bp of each other and 25 of these DMPs were co-methylated in the same direction. Out of the 53 most methylated DMPs, 8 were co-methylated in the same direction. These included *C5orf39* that had 4 DMPs, *FOXP1* and *NRN1* that had 3 DMPs, while *RIOK3* had 2 DMPs within 5 base pairs with β methylation values of +11 and +17% (Table 2a).

We also include the M value, calculated as the log₂ ratio of the intensities of methylated versus unmethylated probes.²⁷ The top M values did not exactly correlate with the β values, but we have categorized the most differentially methylated CpG sites according to β value difference, which reflect biological activity, while M value difference more accurately assess statistical difference. (Table 2a and b).

Potential role of SNPs influencing DMPs

Underlying genetic polymorphisms can influence differences in methylation of CpG sites.²⁸ We assessed the potential influence of SNPs on DNA methylation, by cross-referencing the 53 DMPs within term cord blood that had a β value difference of >10% with more than 150,000 SNPs in the Illumina database of SNPs known to influence methylation (http://support.illumina.com/downloads/infinium_hd_methylation_snp_list.ilmn). We found 13 hypermethylated and hypomethylated positions in close proximity to SNPs. These included a hypomethylated CpG site (β

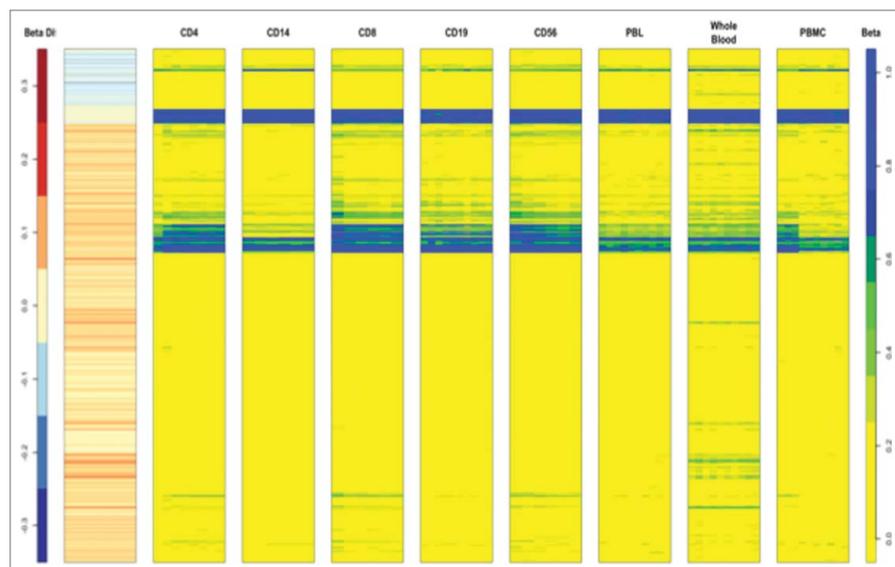


Figure 2. Heat map of the 839 DMPs between FGR and AGA in circulating cord blood cell subtypes identified as CD4, CD14, CD8, CD19, CD56, peripheral blood leucocytes, whole blood or peripheral blood mononuclear cells from 109 different samples. 450K data from a publicly available database Marmal-aid (<http://marmal-aid.org>)²⁵ (see methods). There is very little difference in methylation between the 839 DMPs according to cell subtype. This is portrayed as similar color profiles for each of the cell types (blue, yellow and green) that correspond to the level of β methylation (far column) of the 839 probes.

Table 2a. The most methylated DMPs (β value $>+10\%$) in umbilical cord blood from FGR compared with AGA offspring. Columns show CG identifying site, chromosome number, chromosome coordinate, gene identifier, chromosome number, methylation β value, and adjusted P value for difference in mean β value between FGR and AGA offspring and M value

ID Hypermethylated Positions	CHR	MAPINFO	UCSC Ref Gene	β value difference	P value	M value difference
cg25418748	5	178977236	RUFY1	0.17	4.10E-08	-0.88
cg20796556	18	21032774	RIOK3	0.17	4.32E-08	-1.02
cg17724814	1	155881358	RIT1	0.16	8.41E-07	-0.99
cg07979357	19	14142353	IL27RA	0.15	4.30E-08	-0.92
cg01769037	6	15246613	JARID2	0.14	2.36E-07	-1.08
cg22256960	15	77711686		0.14	2.70E-06	-0.90
cg19857457	18	47018947	RPL17;SNORD58A	0.14	1.92E-07	-0.90
cg27069263	2	55278118	RTN4	0.14	1.57E-05	-0.86
cg18261223	5	32711517	NPR3	0.14	2.76E-06	-1.01
cg09435090	4	17513980	QDPR	0.14	1.17E-06	-0.86
cg20684973	10	105127632	TAF5	0.14	5.18E-08	-1.06
cg00565558	7	6120396		0.14	2.07E-08	-0.82
cg23802518	10	80827482	ZMIZ1	0.14	2.47E-07	-0.91
cg18036763	22	45404910	PHF21B	0.13	9.57E-08	-0.84
cg08965527	16	84178213	HSDL1;LRRC50	0.13	6.78E-08	-0.83
cg10861135	9	14345348		0.13	3.31E-07	-0.91
cg00510787	6	151772946	C6orf211;RMND1	0.13	2.03E-06	-0.78
cg09747578	1	46769076	LRRC41;UQCRH	0.12	9.43E-08	-0.89
cg18022926	10	70093071	PBLD;HNRNPH3	0.12	1.24E-07	-0.88
cg10013169	6	5997028		0.12	1.98E-06	-0.86
cg22276571	20	17549599	DSTN;DSTN	0.12	4.88E-07	-0.77
cg16595484	3	122512170	HSPBAP1	0.12	1.25E-06	-0.71
cg03393426	10	102986686		0.12	1.07E-07	-0.86
cg04921814	1	145575587	PIAS3	0.12	2.26E-06	-0.87
cg21935083	5	131892314	RAD50	0.12	5.42E-07	-0.64
cg23653187	22	44319257	PNPLA3	0.12	8.65E-07	-0.88
cg17344340	22	46044084		0.11	7.95E-06	-0.68
cg07772516	15	52107742	TMOD2;TMOD2	0.11	6.76E-06	-0.67
cg08779777	7	106505772	PIK3CG	0.11	2.62E-05	-0.72
cg21860429	6	105389544		0.11	2.19E-05	-0.67
cg14555127	7	35841578	SEPT7;	0.11	4.86E-07	-0.76
cg04609859	17	46655736	HOXB4	0.11	1.45E-06	-0.77
cg21312090	8	116681727	TRPS1	0.11	2.44E-06	-0.74
cg17373554	5	72594735		0.11	1.88E-05	-0.67
cg05489143	3	40498640	RPL14	0.11	8.96E-07	-0.81
cg04005701	17	1733641	RPA1;SMYD4	0.11	1.87E-07	-0.70
cg20760063	17	41277580	NBR2;BRCA1	0.11	4.40E-08	-0.85
cg06361531	16	68057779	DUS2L;DDX28	0.11	2.72E-07	-0.69
cg01013600	19	49141177	DBP;SEC1	0.11	3.42E-07	-0.71
cg25845597	6	27841122	HIST1H4L;HIST1H3I	0.11	2.00E-07	-1.08
cg14036868	2	38604442	ATL2	0.11	7.45E-07	-0.95
cg26220528	12	57623348	SHMT2	0.11	5.92E-07	-0.80
cg01147107	1	47696505	TAL1	0.11	5.12E-07	-0.70
cg16942681	18	21032779	RIOK3	0.11	6.05E-05	-0.74
cg08215925	3	71633214	FOXP1	0.11	1.02E-05	-0.98

Table 2b. Table shows the most hypomethylated DMPs (β value $>-10\%$) in umbilical cord blood from FGR compared with AGA offspring. Columns show CG identifying site, chromosome number, chromosome coordinate, gene identifier, chromosome number, methylation β value, and adjusted P value for difference in mean β value between FGR and AGA offspring and M value

ID	CHR	MAPINFO	UCSC Ref Gene	β value	P value	M value difference
cg23372001	6	27791640	HIST1H4J	-0.22	1.10E-10	1.83
cg26758857	22	36649135	APOL1	-0.18	3.47E-07	1.18
cg05533953	4	331685	ZNF141	-0.15	1.69E-10	1.45
cg26542283	5	43040505	C5orf39	-0.15	2.77E-09	1.66
cg07875360	5	1801344	NDUFS6;MRPL36	-0.13	4.60E-08	0.95
ch.20.327316F	20	15628644	MACROD2	-0.12	8.34E-10	0.74
cg01458605	3	147128679	ZIC1	-0.11	4.69E-10	1.00
cg04410715	19	39283334		-0.11	2.53E-08	0.83

value -0.15) within 7 and 24 base pairs of 2 *C5orf39* SNPs and one hypermethylated site (β value +0.14) within 47 base pairs of a *TAF5* gene variant.

Furthermore, within the 839 DMPs found in umbilical cord blood between FGR and AGA at term, we did not observe the methylation pattern typically associated with SNPs (0%, 50%, or 100%). It is also of note that cord blood and placenta are of fetal origin and share the same genotype, but we found no overlap in DMPs between these tissues. Taken together, these observations suggest that the methylation differences between FGR and AGA offspring that we found are unlikely to be driven by SNPs.

Functional pathways of DMPs discovered in cord blood between FGR and AGA offspring using the Marmal-aid database

The Marmal-aid DNA methylation database, which contains >10,000 samples in one repository,²⁵ was used to gain insight

into the functionality of the 839 differentially methylated CpG sites that we discovered in cord blood from FGR offspring born at term. The Marmal-aid output suggested that the 839 DMPs are found in normally very stable unmethylated or hypomethylated gene loci consistent across all blood cell types. Also, these same positions vary little in methylation status between tissue type and through aging and are located in highly conserved, invariable regions of the genome.

To establish whether the 839 term DMPs linked to Ref Seq-annotated genes had significant functional roles, we used GO pathway analysis. Using an R based package and thresholds described in the materials and methods section, 23 GO pathways were identified (Table 3). Many of these pathways contained DMPs with >10% β value differences between FGR and AGA offspring, supporting a potential functional role for these DMPs. The majority of pathways identified through GO analysis related to regulation of gene transcription and expression and control of metabolic processes. Pathways with the greatest methylation

Table 3. Twenty-three GO pathways identified using 839 DMPs within FGR term umbilical cord blood samples. Fold change refers to the number of DMP on genes actually present in the pathway compared with the number expected and that met the P -value < 0.0001 and cut off of 2-fold change (rounded up). The last 2 columns report the most differentially methylated genes (>10%) found in the respective GO pathway.

GO term	Fold change	Gene in pathway >10% β value difference	
		Hypermethylated	Hypomethylated
DNA recombination	3.05	IL27RA, FOXB1, RAD50	
cellular process	2.6	RIOK3; IL27RA; RIT1; JARID2; TAF5; RAD50; HOXB4; TRPS1; RPL14; ATL2; FOXP1	APOL1; ZNF141; MACROD2; ZIC1
regulation of transcription from RNA polymerase II promoter	2.32	JARID2; HOXB4; TRPS1; FOXP1	
transcription from RNA polymerase II promoter	2.23	JARID2; TAF5; HOXB4; TRPS1; FOXP1	ZNF141
embryo development	2.15	HOXB4; FOXP1	ZIC1
central nervous system development	2.15	JARID2; FOXP1	ZIC1
positive regulation of transcription from RNA polymerase II promoter	2.09	FOXP1	
regulation of nucleobase-containing compound metabolic process	2.03	IL27RA; JARID2; TAF5; RAD50; HOXB4; FOXP1	ZNF141; ZIC1
negative regulation of nucleobase-containing compound metabolic process	2.01	JARID2; HOXB4; FOXP1	
regulation of nitrogen compound metabolic process	2.00	IL27RA; JARID2; TAF5; HOXB4; TRPS1; FOXP1	ZNF141; ZIC1
RNA biosynthetic process	2.00	TRPS1; FOXP1	ZIC1
negative regulation of gene expression	1.99	JARID2; HOXB4; TRPS1; FOXP1	
negative regulation of cellular macromolecule biosynthetic process	1.99	JARID2; HOXB4; TRPS1; FOXP1	
regulation of cellular macromolecule biosynthetic process	1.98	JARID2; HOXB4; TRPS1; FOXP1	ZNF141; ZIC1
regulation of transcription, DNA-dependent	1.98	JARID2; TAF5; HOXB4; TRPS1; FOXP1	ZNF141; ZIC1
negative regulation of nitrogen compound metabolic process	1.98	IL27RA; JARID2; TAF5; HOXB4; TRPS1; FOXP1	ZNF141; ZIC1
regulation of gene expression	1.97	ZIC1; ZNF141; HOXB4; FOXP1; JARID2; TAF5	ZNF141; ZIC1
transcription, DNA-dependent	1.97	JARID2; TAF5; HOXB4; FOXP1	ZNF141; ZIC1
regulation of RNA biosynthetic process	1.97	JARID2; TAF5	
organ morphogenesis	1.97	HOXB4; FOXP1	ZIC1
nucleic acid metabolic process	1.96	IL27RA; JARID2; TAF5; HOXB4; FOXP1	ZNF141; ZIC1
regulation of macromolecule biosynthetic process	1.96	JARID2; TAF5; HOXB4; FOXP1	ZNF141; ZIC1
negative regulation of macromolecule biosynthetic process	1.96	JARID2; HOXB4; TRPS1; FOXP1	
regulation of RNA metabolic process	1.96	JARID2; TAF5; HOXB4; FOXP1	ZNF141; ZIC1

difference were involved in the regulation and transcription of RNA polymerase II.

Placental DMPs between FGR and AGA offspring

In the placenta from term deliveries, there were no DMPs between FGR and AGA offspring that reached genome-wide significance. To elucidate whether the 453 placental DMPs initially identified between FGR and AGA offspring were primarily driven by differences in gestational age, we ran a linear model on all placental samples using gestational age as a continuous variable across the gestational age range (202 to 299 days). We identified 20,893 placental DMPs associated with gestational age, heavily influenced by placentae from early gestational FGR pregnancies. The majority of placental DMPs were hypomethylated (93%), but with advancing gestation the placenta became more methylated (adjusted $P < 0.05$), consistent with the findings of others.¹⁷

We validated placental results using methylation data from a study of placental tissue from healthy pregnancies, across all 3 trimesters and run on an Illumina HumanMethylation27 BeadChip, a precursor of the 450K array.¹⁷ Using the same cutoff for statistical significance as our study (FDR-adjusted $P < 0.05$) we identified placental DMPs between the second trimester and term groups in the published data set¹⁷ and searched for similar placental DMPs between the second trimester and term in our own cohort. Out of 23,677 positions available for direct comparison between the 27K and 450K platforms, 825 DMPs were called from these positions using the 450K analysis and of these, 541 (65%) were also identified using the 27K analysis.¹⁷ Both data sets contained 519 similarly hypomethylated and 13 similarly hypermethylated positions. Only 9 probes showed methylation in opposite directions (Fig. 3). The remaining 284/825 DMPs unique to our FGR cases have the potential to have a role in fetal growth independent of gestational age. GO pathway analysis of these 284 placental DMPs showed 16 pathways that met the criteria for reporting. These included pathways involved in the regulation of autophagy, response to oxidative stress and hormonal stimuli (Table 4).

Discussion

We found that cord blood from newborn offspring affected by fetal growth restriction (FGR) had significant DNA methylation differences compared with appropriately grown offspring (AGA). Methylation differences persisted after accounting for differences in gestational age and were consistent across leucocyte populations in umbilical cord blood.

DMPs were associated with genes involved in pathways crucial to key cellular processes, particularly regulation of gene expression and transcription. Several of the 53 DMPs with the greatest methylation differences were methylated at more than one CpG site, emphasizing the potential for methylation differences to influence gene expression.

Three of the most differentially methylated positions between FGR and AGA cord blood were co-methylated at several CpG sites on genes that control ubiquitous transcription factors. *FOXP1*, which was hypermethylated at 3 CpG sites, is expressed in many tissues and is important for organogenesis, metabolism, and immunity.²⁹ *RIOK3* was hypermethylated on 2 CpG sites just 5 base pairs apart (β values +11% and +17%). *RIOK3* is an atypical protein kinase that interacts with caspase-10 and negatively regulates NF-Kappa B.³⁰ Inhibition of NF-Kappa B reduces angiogenesis, but permits apoptosis with the potential to compromise fetal growth and development.³¹ *TAF5* encodes a key subunit that is critical to the formation of basal transcription factor, Transcription Initiation Factor TFIID subunit 5 (TFIID).³² TFIID is used by RNA polymerase II for transcript initiation and is critical for reprogramming of pluripotent embryonic stem cells.³³ Enhanced expression of TFIID during fetal life may therefore generate plasticity in gene expression that could influence embryonic stem cells to pass into different cellular states. RNA polymerase II pathways also featured heavily in our GO analysis of term DMPs in cord blood from FGR offspring. Epigenetic interference with the expression of these transcription factors has the potential for far reaching effects during a time of developmental plasticity.

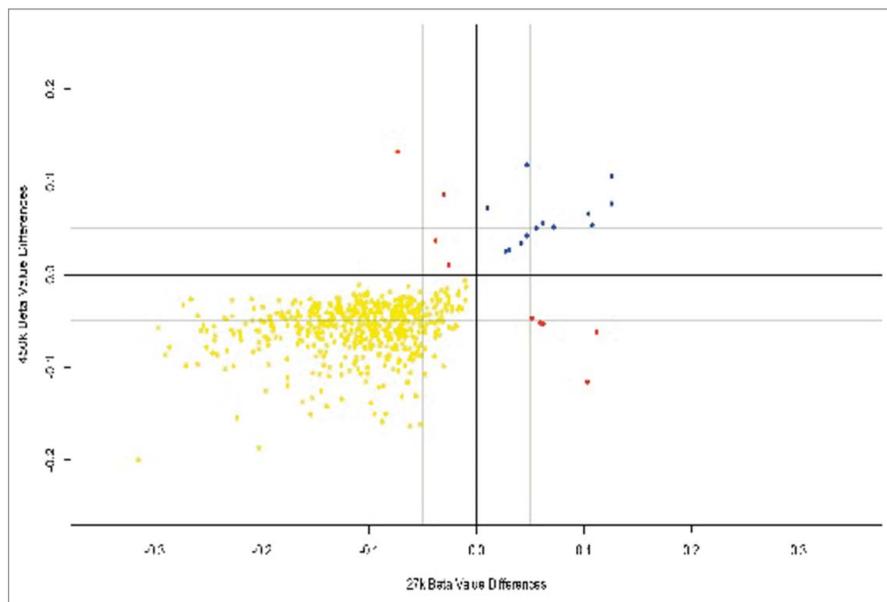


Figure 3. Placental DMPs between 2nd and 3rd trimesters in our data set using the Infinium HumanMethylation450 Beadchip® and also present in an external data set using the Infinium HumanMethylation27.¹⁷ There were 519 hypomethylated DMPs common to both platforms (yellow), 13 hypermethylated DMPs common to both platforms (blue) and 9 DMPs that were mismatched with regards direction of methylation (red).

Table 4. GO pathway analysis of 284 placental DMPs associated with our FGR cohort. These DMPs were identified after validation analysis with an external data set of placentas from healthy pregnancies across 3 trimesters.¹⁷ After excluding DMPs that were present on both platforms and assumed to be associated with gestational age, the remaining 284 DMPs remained unique to our FGR cohort. Sixteen functional pathways met the criteria for reporting. Pathways known to be associated with fetal growth restriction and that were evident in the placenta of our FGR cohort included autophagy, response to oxidative stress and the regulation of monocyte chemotaxis. The actual and expected number of DMPs on genes in the pathway is recorded and the fold change between these counts that met the P -value <0.0001 and cut off of >2 -fold change

GOBPID	Fold change	Exp count	Actual count	GO Term
GO:0016239	31.4	0.14	3	positive regulation of macroautophagy
GO:0090026	31.4	0.14	3	positive regulation of monocyte chemotaxis
GO:0010508	16.8	0.3	4	positive regulation of autophagy
GO:0032312	12	0.4	4	regulation of ARF GTPase activity
GO:0010506	9.78	0.72	6	regulation of autophagy
GO:0006914	5.12	1.49	7	autophagy
GO:0043627	4.24	2.31	9	response to estrogen stimulus
GO:0006979	4.08	3.5	13	response to oxidative stress
GO:0031667	3.06	5.31	15	response to nutrient levels
GO:0032870	3.05	6.07	17	cellular response to hormone stimulus
GO:0043434	3.05	5.71	16	response to peptide hormone stimulus
GO:0051270	2.85	6.07	16	regulation of cellular component movement
GO:0009991	2.85	5.68	15	response to extracellular stimulus
GO:0009725	2.49	10.6	24	response to hormone stimulus
GO:0071495	2.46	8.35	19	cellular response to endogenous stimulus
GO:0032879	2.04	18.4	34	regulation of localization

A minority of DMPs on genes from FGR cord blood were hypomethylated compared with AGA offspring. Gene variants of *APOL1*, which we found to be hypomethylated (β value, -18%) are associated with accelerated kidney disease and lower HDL concentrations, especially in African Americans.^{34,35} Methylation differences on *APOL1* were also found in cord blood mononuclear cells in twins and correlate with birth weight.³⁶ Interestingly, a near neighbor of *APOL1* on chromosome 22 is *PNPLA3*, which we found to be hypermethylated (β value $+0.12$). Gene variants of *PNPLA3* produce excess adiponutrin, which promotes lipid synthesis and increases the risk of non-alcoholic fatty liver disease.³⁷

We also found hypomethylation on *C5orf39* (β value -15% and co-methylated in 4 positions) is a gene unique to humans that encodes Annexin II receptor, a novel activator of apoptosis.³⁸ Low birth weight offspring show increased lymphocyte apoptosis, which correlates with insulin resistance.³⁹

Genes that encode histone proteins were found to be differentially methylated in both directions, *HIST1H4J* (β value -22%) and *HIST1H3I* (β value $+11\%$). There were 7 DMPs regulating histone modification, which may suggest a role for epigenetic events other than DNA methylation influencing gene expression in FGR offspring. Histone modification, which most often involves post-translational changes to the tail of a histone protein, influences transcription of the associated gene and has a role in human development.⁴⁰ Cellular pluripotency during fetal life is partly determined by histone modification and therefore factors that influence histone gene expression may play an important role in this critical element of fetal development.⁴¹

Umbilical cord blood from over 1000 infants in the Norwegian Mother and Child Cohort assessed using the Infinium HumanMethylation450 BeadChip showed differential methylation at 19 CpGs associated with either increased or decreased

birth weight across a range of normal birth weights.⁴² Two genes, *ARID5B* and *XRCC3*, were particularly associated with either decreased or increased birth weight, respectively. These genes did not appear among the strongly differentially methylated genes in our cohort of pathologically growth restricted offspring. It is possible that the influence of DNA methylation is different within a spectrum of healthy birth weight offspring as compared with pathological growth restriction.

We studied well-phenotyped FGR offspring identified in utero according to customized fetal growth scales in otherwise healthy mothers and in the absence of other fetal disease. In all but 1 of the 29 cases for whom we have data, there was elevated utero-placental perfusion pressures with poor placental perfusion, before the identification of FGR. In an animal model that mimics human fetal growth restriction by ligating both uterine arteries, epigenetic dysregulation was seen at conserved intergenic sequences in 7-week old offspring.⁴³ These epigenetic events were near genes that regulate vascularization, pancreatic β cell proliferation, and apoptosis, which may underpin the vulnerability of FGR offspring to type-2 diabetes.³³ Similarities between this animal model and the placental driven FGR in our cases, supports the concept that the methylation differences seen in FGR offspring are secondary to reduced utero-placental perfusion.

Animal models have also shown that maternal and paternal diet can alter offspring phenotype through epigenetic mechanisms.^{44,45} We studied FGR offspring from well-nourished mothers who had a similar phenotype to mothers of AGA offspring, but in whom there was poor placental development. Interestingly, we found sub-clinical insulin resistance in the fathers of FGR pregnancies, as we previously reported.⁴⁶ We did not assess the paternal epigenome, but animal studies support the possibility that paternal diet-induced epigenetic

change can be inherited by offspring and lead to reduced fetal growth.⁴⁵ It is also possible that gene variants that predispose the father to insulin resistance are inherited by offspring. Under these circumstances, insulin cannot act as an effective growth factor in utero and predisposes the fetus to diabetes in later life.⁴⁷

To date, the study of rare imprinted gene disorders has shown an influential role for altered methylation of genes essential to fetal growth.^{19,48} Our study has taken a broader, unbiased whole-genome approach to identify methylation changes on genes of FGR offspring secondary to poor placental development. In our cases, we found low-level methylation differences in 2 imprinted genes *Plagl1* and *Kcnq1*, but despite good coverage in the HM450 data set, we found no differences in methylation of *Igf2/H19*. Our study suggests that imprinted genes have little influence on FGR secondary to poor placental development, but rather genes encoding key transcription factors are differentially methylated. Future studies might investigate the parent of origin of these novel regions of differential methylation.

Fetal growth restriction often results in iatrogenic premature childbirth, secondary to failure of the fetus to thrive in utero. This leads to the dual problems of low birth weight and prematurity. In our study we dissected apart the influence of gestational age from impaired fetal growth by matching FGR cases that delivered at term with the AGA offspring and by excluding DMPs associated with increasing gestational age. We also compared our placental methylation results with a published data set.¹⁷ Although we showed methylation status of the placenta and cord blood altered with advancing gestational age, we identified DMPs that remained unique to FGR offspring. Further investigation of the effects of these DMPs on placental gene function is necessary to understand how they might influence fetal growth and predispose to disease in later life.

In placentas of pre-term FGR offspring, GO pathway analysis identified differential methylation on genes implicated in pathways important to oxidative stress, hormonal stimulation, and autophagy. These pathways are crucial to normal placental development, while abnormal autophagy and oxidative stress are recognized features of FGR placentas.⁴⁹⁻⁵¹

At term, we did not observe methylation differences between the placentas from FGR and AGA that were evident in earlier pregnancy. It is possible that given time, pathological differences in the placental methylome that influence fetal growth give way to gestational influences in anticipation of childbirth. Differences in placental methylation in pregnancies destined to result in fetal growth restriction appear to be more pronounced in early pregnancy.

The Infinium HumanMethylation27 BeadChip[®] platform, was previously used on 206 term placentas and identified 22 methylation loci that could act as markers for poor fetal growth.⁵² Notably, placentas from growth restricted (FGR) and constitutionally small offspring (SGA) were combined in this analysis.⁵² Although we did not replicate methylation loci using the Infinium HumanMethylation450 BeadChip[®] with this cohort, methylation differences on pathways associated with basic

cellular functions including transcription and DNA repair⁵² were similar to those we found in pre-term placentas. However, 6 of the 22 DMPs in the FGR and SGA cohort combined⁵² were present in the second and third trimester of the external placental dataset,¹⁷ supporting a possible influence of these loci on gestational age.

Placentas from pregnancies affected by pre-term preeclampsia, a condition that shares very similar placental characteristics to FGR pregnancies,⁵³ have been found to have distinct methylation profiles using the 450 BeadChip.⁵⁴ However, no further differences in placental methylation were found according to whether the preeclamptic pregnancies were also affected by FGR or AGA offspring.⁵⁴ The differences we found in methylation profile in umbilical cord blood between FGR and AGA suggests that the fetal response to the same placental pathology is distinctive for those who cannot grow to their full potential.

Placentas from monozygotic twins discordant for fetal growth, have shown increased DNA methylation of the leptin promoter region and upregulation of leptin mRNA in the smaller twin.⁵⁵ Similarly, expression of *LEPROTI* (Leptin receptor overlapping transcript-like 1) from cord blood mononuclear cells of dichorionic twins showed a negative correlation with birth weight.⁵⁶ These observations may explain the future vulnerability of low birth weight offspring to metabolic syndrome. However, we did not find any DMPs associated with leptin that reached genome wide significance between FGR and AGA placentas.

Although there was no difference in the mode of delivery of FGR and AGA offspring born at term, the mode of delivery has been shown to influence offspring methylation status.⁵⁷ Offspring born by caesarean section have higher DNA methylation in umbilical cord blood leucocytes compared with those born by vaginal delivery.⁵⁷ Interestingly, methylation differences associated with mode of delivery did not persist when re-examined in neonatal leucocytes 3–5 d postpartum.⁵⁷ It is possible that transient environmental exposure such as mode of delivery causes short-lived epigenetic changes, while persistent environmental influences during a time of high developmental plasticity in utero, cause more enduring effects.

A potential limitation of our study was that we assessed DNA methylation in umbilical cord whole blood at birth, while fetal circulating cell types change according to birth weight and gestation at delivery.⁵⁸ However, at term nucleated red blood cells comprise a small percentage of total cell number (3 per 100 wbc), which limits their influence on the methylation of cells from cord blood.⁵⁹ Furthermore, according to the Marmalaid data set, we showed a similar pattern of DMPs in areas normally unmethylated and across cord blood cell types. Differences in cell type that might exist at term between FGR and AGA are unlikely to play a significant role in observed DMPs.²⁶ Cell composition is however likely to explain some of the 76,249 DMPs that we identified in cord blood of pre-term FGR offspring.

Despite many human studies investigating genetic and environmental mechanisms that might link low birth weight with risk of adult disease, there is very little reproducible evidence to support a unifying pathophysiological process. This may partly reflect the multi-factorial elements that lead to FGR as well as

subsequent environmental influences that promote vulnerability to polygenic disease, such as type-2 diabetes mellitus (T2DM). Although gene variants can have pleiotropic effects on different organs, organ specific diseases need to be investigated using the relevant tissue. In this regard, the study of the placenta is relevant to fetal growth restriction, but the study of less accessible tissue, such as the pancreas, is needed to investigate the metabolic syndrome or T2DM. Altered methylation status of selected candidate genes from umbilical cord tissue has however been correlated with later life phenotype.¹² Conversely, saliva from monozygotic monochorionic twins aged 34 years, showed very similar genome-wide DNA methylation profiles between twins that were discordant for birth weight.¹⁵

The novel cord blood DMPs in well-phenotyped FGR offspring that we observed warrant further targeted study that might provide a pathophysiological link between low birth weight and adult disease. DMPs in FGR cord blood may be a consequence of placental dysfunction or inherited from insulin resistant fathers. Reassessment of FGR neonates in adulthood would determine if DMPs identified at birth have endured into later life to influence vulnerability to adult disease.

Patients and Methods

Selection of cases and controls

A case-control collection of samples from pregnancies affected by fetal growth restriction and appropriately grown offspring was undertaken at University College London Hospital (UCLH) between September 2009 and May 2011. The Joint UCLH/UCL (α) Ethical Committee (09/H0715/28) granted ethical approval for the study. All participants gave informed consent. Caucasian women were included as cases when an ultrasound scan identified fetal growth restriction (FGR), defined as an estimated fetal weight <10th customized centile for gestational age and adjusted for maternal weight, height, parity, and ethnicity (available from Perinatal Institute, UK - www.gestation.net). Pregnancies were excluded if fetal growth restriction was associated with fetal structural or chromosomal abnormalities, infections, chronic maternal disease, or multiple pregnancies. Pregnant women were included in the control group if they were found to have an appropriately grown for gestational age (AGA) fetus, with an estimated fetal weight between 10th and 95th customized centile, born at more than 255 d and only if predicted normal fetal growth matched neonatal measures after delivery. After a diagnosis of FGR, we examined case notes for information on uterine artery blood flow at 20–22 week gestation.

At childbirth, umbilical cord blood was taken from the umbilical vein, and stored at -80°C . Placental samples were taken from the fetal surface within 2 cm of the cord insertion and stored at -80°C . Gestational age, offspring gender, weight, length, and delivery details were recorded.

Within 4 weeks of childbirth, paternal phenotype was also assessed. Specifically, we measured paternal blood pressure, waist circumference, fasting glucose and insulin levels.

DNA extraction

DNA was extracted from paired placenta and cord blood samples using standard kits and protocols (DNeasy Blood & Tissue Kit, Qiagen, UK). After cleanup (DNA Clean & Concentrator Kit, Zymo Research, Cambridge Biosciences, UK), samples with a 260/280 ratio >1.75 and a 260/230 ratio >1.8 were discarded from further analysis.

Bisulphite conversion

High-quality genomic DNA (500ng) was bisulphite converted using the EZ-96 DNA Methylation Deep-well Kit (Zymo Research, Cambridge Biosciences, UK). Bisulphite conversion efficiency was assessed using qPCR with primers to converted and non-converted DNA (Primers and cycling parameters in appendix) and samples that showed $<97\%$ bisulphite conversion were excluded. A total of 56 cord blood and 54 placental samples were suitable for methylation analysis, including 49 participants with both cord blood and placental samples and 12 further tissue samples (7 cord blood and 5 placenta).

Illumina human methylation450 beadChip

Bisulphite-converted samples were hybridized to the Illumina HumanMethylation450 BeadChip (Illumina, Inc., CA, USA)⁶⁰ using standard protocols. Cases and controls and tissue type were arranged randomly on each chip and processed in a blinded fashion. Arrays were scanned using the Illumina iScan SQ 2-color laser scanner.

Quality Control

We evaluated the quality of the data based on the signals of control probes built in to the assay. This resulted in 90 samples that were suitable for downstream analysis. Multiple Dimensional Scaling analysis (MDS) demonstrated no significant positional or chip batch effect between samples.

Quantile normalization was performed separately for individual color channels, probe types, and tissue types (cord blood and placenta) due to their variable distribution of methylation values (β values). Probe β values (methylated probe intensity/[methylated probe intensity + unmethylated probe intensity + 100]) were converted to M-values by Logit transformation to reduce heteroscedasticity.²⁷

Of 486,428 probes present on the array, 11,648 X and Y chromosome probes were removed; 1,927 were removed for failing to meet the detection P -value cut-off ($P = 0.05$) and a further 43,097 were removed due to probe cross-hybridization. This resulted in a total of 429,756 probes, representing individual positions of methylation available for downstream analysis.

Statistical Analysis

The influence of gestational age on DMPs between FGR and AGA was assessed using a continuous linear regression model in the Limma package, incorporating all samples and gestational

age. Results were validated against an external data set of placental tissue from AGA pregnancies that were investigated using the Illumina 27K methylation array.¹⁷

To further characterize the DMPs, we utilized Marmal-aid (<http://marmal-aid.org>),²⁵ a recently developed meta-analysis tool. In Marmal-aid, all publicly available Illumina 450K data (>10,000 different arrays as of June, 2013) is incorporated into a single repository and re-processed to enable customizable meta-analyses.

GO pathway analysis was also performed in order to deduce gene function of identified DMPs. We only reported those pathways that had a 2-fold (rounded up) enrichment of genes more than expected and that reached a significant *P*-value of <0.0001.

Author Contributions

SH, SF, VR, GH, and DW designed the study. SH recruited patients and collected samples. SH and MS extracted DNA, bisulphite converted and performed QC on DNA. SH, CM, RL,

and VR carried out the bioinformatics analysis. All authors contributed to interpretation of the data. SH and DW wrote the first and last drafts of the manuscript and all authors made critical revisions. GH and DW take responsibility for the integrity of the data and accuracy of the data analysis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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