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Title page

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Consent to participate and for publication: Written consents were obtained from all participants of the IOW and Born Into Life cohorts at recruitment and follow-ups.

Availability of data and material: Data used in this study may be available upon request from the authors with permission from the IOW and Born Into Life cohorts.

Code availability: N/A

Abstract

This study aims to evaluate the association of maternal DNA methylation (DNAm) during pregnancy and offspring birthweight. 122 newborn-mother dyads from the Isle of Wight (IOW) cohort were studied to identify differentially methylated cytosine-phosphate-guanine sites (CpGs) in maternal blood associated with offspring birthweight. Peripheral blood samples were drawn from mothers at 22–38 weeks of pregnancy for epigenome-wide DNAm assessment using the Illumina Infinium HumanMethylation450K array. Candidate CpGs were identified using a course of 100 repetitions of a training-and-testing process with robust regressions. CpGs were considered informative if they showed statistical significance in at least 80% of training and testing samples. Linear mixed models adjusting for covariates were applied to further assess the selected CpGs. The Swedish Born Into Life cohort was used to replicate our findings (n=33).

Eight candidate CpGs corresponding to the genes *LMF1*, *KIF9*, *KLHL18*, *DAB1*, *VAX2*, *CD207*, *SCT*, *SCYL2*, *DEPDC4*, *NECAP1*, and *SFRS3* in mothers were identified as statistically significantly associated with their children's birthweight in the IOW cohort and confirmed by linear mixed models after adjusting for covariates. Of these, in the replication cohort, three CpGs (cg01816814, cg23153661, and cg17722033 with p-values= 0.06, 0.175, and 0.166, respectively) associated with four genes (*LMF1*, *VAX2*, *CD207*, and *NECAP1*) were marginally significant.

Biological pathway analyses of three of the genes revealed cellular processes such as endocytosis (possibly sustaining an adequate maternal-fetal interface), and metabolic processes such as regulation of lipoprotein lipase activity (involved in providing substrates for the developing fetus). [Our results contribute to an epigenetic understanding of maternal involvement in offspring birthweight. Measuring DNAm levels of maternal CpGs may in the future serve as a diagnostic tool recognizing mothers at risk for pregnancies ending with altered birthweights.](#)

Keywords

DNA Methylation; Epigenetics; Epigenome-Wide Association Study; Pregnancy; Birthweight

Introduction

Birthweight is an important indicator of pregnancy outcome and neonatal health. Both low and high birthweight categories have been linked to adverse health outcomes.[1-4] Maternal factors such as cigarette smoking, hyperglycemia, and hypertension have been documented to affect the offspring birthweight.[5] It has been suggested that effects of maternal factors during pregnancy on birthweight are exerted through differential methylation of DNA of offspring exposed to the intrauterine environment.[6] A meta-analysis of epigenome-wide studies on birthweight and neonatal DNA methylation (DNAm) reported that 914 cytosine-phosphate-guanine sites (CpGs) are differentially methylated in association with birthweight.[5] However, due to concurrent measurements of birthweight and DNAm, it remains unclear whether differentially methylated sites represent effects of lower birthweight or risk factors for differences in birthweight.

No study in the literature has yet assessed the associations of maternal DNAm in pregnancy and offspring birthweight. Maternal DNAm may influence birthweight of the offspring by impacting expression of the maternal genes that are important for fetal development. A recent study that investigated DNAm at age 18 years to early pregnancy showed that changes in maternal DNAm may be located on genes belonging to signaling pathways controlling uterus and trophoblast interaction such as cell adhesion and cell division.[7] Considering the critical role of in-utero environment in fetal development, we postulated that differential maternal DNAm during gestation could affect the offspring's growth and birthweight. In this study, we investigated the association of maternal DNAm during pregnancy and offspring birthweight. In the discovery step, we identified differentially methylated CpGs related to birthweight, using the 3rd generation Isle of Wight (IOW) cohort. Then, in an independent mother-offspring cohort, the Swedish Born Into Life cohort, we tested whether we could replicate our findings. In addition, to assess if

maternal DNAm affects the gestational age, not the birthweight, we tested the potential mediating role of gestational age between maternal DNAm and birthweight.

Materials and Methods

Study population

IOW cohort

The IOW cohort was established in 1989 in UK to investigate the natural history of allergic disorders and asthma.[8] The IOW study was approved repeatedly by the local research ethics committee (NRES Committee South Central – Hampshire B, U.K.) and the University of Memphis Institutional Review Board in Memphis, U.S. (FWA00006815). Written consents were obtained from all participants at recruitment and all follow-ups. The IOW cohort consists of three generations (F0-parents of the original birth cohort, F1- birth cohort members, and F2-offspring of F1). The current analysis focuses on adult female IOW study participants (F1) and their offspring (F2). The F2 generation is comprised of 542 newborns from 331 mothers. Excluding subjects with missing data on birthweight, gender, or maternal DNAm, a total of 122 newborns of 114 mothers (8 mothers with 2 newborns) remained for analysis.

Peripheral blood samples were drawn from F1 participants during the first (8–21 weeks) and the second (22–38 weeks) halves of pregnancy for epigenome-wide DNAm assessment. After delivery, umbilical cord blood samples were collected from F2 participants. Maternal anthropometrics (weight, height, BMI) were measured early in pregnancy. Information on F1 such as parity, smoking during pregnancy, and socioeconomic status was ascertained by questionnaires. Smoking during pregnancy was classified as none, light (between 1 and 9 cigarettes per day), and moderate smoking (at or above 10 cigarettes per day). Socioeconomic

status is a composite variable derived from three indicators: the British socioeconomic classes based on parental occupation (1-6), number of children in the index child's bedroom, and family income.[9] The derived variable was categorized into three levels: low, medium, and high, respectively. The proportions of maternal blood cell-types (neutrophil, eosinophil, B cell, CD4+ T cell, natural killer cell) were estimated using the “minfi” R packages [10] based on reference values of cell-type-specific CpGs. [11] Newborns' gender, gestational age, and birthweight were transcribed from hospital records.

Born into life cohort

The Born into Life cohort focuses on effects of maternal factors and early biomarkers during pregnancy on child's growth and health outcomes later in life in Sweden. This study is described in a previous publication [12]. The data contribution from this study consisted of 33 women with available DNAm data during late pregnancy (weeks 26-28). Ethical approval was obtained by the Regional Ethics Review Board in Stockholm, Sweden, and written consents were obtained from each subject in the Born into Life cohort. Maternal peripheral blood samples were collected for epigenome-wide DNAm assessment at gestational weeks 26 to 28.

DNAm profiling

DNA from maternal peripheral blood and umbilical cord blood was extracted by a standard salting out procedure.[13] Using the EZ 96-DNA methylation kit (Zymo Research, CA, USA), about 1 µg of DNA was bisulfite-treated for conversion of cytosine to thymine according to the manufacturer's standard protocol in all samples. DNAm was assessed by the Illumina Infinium HumanMethylation450 Beadchip (Illumina, Inc., CA, USA). A standard protocol was used to process arrays.[14] To control for batch effects, samples were randomly allocated on

microarrays. The beadchips were scanned by a BeadStation. We used Bioconductor packages IMA [15] and ComBat [16] for preprocessing the methylation data and removal of batch effect, respectively. Methylation level (β value) was determined for each CpG locus using the Methylation module of BeadStudio software. Beta values ($\beta = \frac{\text{methylated}}{\text{methylated} + \text{unmethylated} + c}$) indicate the proportions of methylated over the sum of methylated and unmethylated sites and c as a constant to prevent dividing by zero. The M-value was calculated as the log₂ ratio of the intensities of methylated sites versus unmethylated sites.[17]

In the replication cohort, DNAm was assessed from peripheral whole blood using DNA extracted at the Karolinska Institutet Biobank. Per sample, an aliquot of DNA (500 ng) underwent bisulfite conversion using the EZ-96 DNA Methylation kit (Zymo Research Corporation, Irvine, USA). Samples were randomized and plated onto 96-well plates and processed with the MethylationEPIC BeadChip, using the standard protocol from the manufacturer (Illumina Inc., San Diego, USA) at the Mutation Analysis Facility, Karolinska Institutet (www.maf.ki.se). This chip measures 866,836 CpG sites across the genome. Methylation data was processed using GenomeStudio Software. The ComBat package was used to remove batch effects.

Statistical analysis

Discovery phase (IOW cohort)

To conduct an epigenome-wide association study (EWAS) identifying maternal CpGs associated with offspring birthweight, the ttScreening R package (v1.5, <http://cran.r-project.org/web/packages/ttScreening/>) [18] was used. This method removes non-informative CpGs in a course of 100 repetitions of a training-and-testing process with robust regressions. CpGs were considered informative if they showed statistical significance in at least 80% of

training and testing samples (selection probability $\geq 80\%$). The ttScreening method has the advantage of detecting more truly positives than conventional EWAS methods.[18] For maternal DNAm levels in ttScreening, we used M values due to having a higher power in detecting highly methylated and unmethylated CpG sites.[17] Following the screening, to assess the association of birthweight with methylation of the candidate CpGs, linear mixed models (in SAS 9.4) were applied to adjust for repeated measurements of maternal DNAm for the eight mothers with two offspring. In these models, we also adjusted for potential confounders (newborn's gender, maternal peripheral blood cell-types, maternal BMI, smoking during pregnancy, and socioeconomic status). Since gestational age was correlated with birthweight ($r=0.56$, p -value $<.0001$) and may act as mediator, it was not used as a confounder in the regression models. However, we ran structural equation modeling in SAS 9.4 to examine the relationship between maternal DNAm at the candidate CpGs, birthweight, and gestational age. To assess the variation of DNAm levels of a CpG from age 18 to early and late pregnancy, we used linear mixed models, with DNAm levels as outcome and time as predictor. Multiple testings in all analyses, including EWAS and associations of informative CpGs with birthweight, were adjusted by applying the false discovery rate (FDR) method.[19] An FDR adjusted p -value of ≤ 0.05 was considered statistically significant.

Replication phase (Born Into Life cohort)

Linear regression models were used to assess the association of candidate CpGs with birthweight in the Born Into Life cohort. Models were adjusted for newborns' gender. Additional factors such as parity and maternal age at delivery were not significant in the model, hence, not included in the analysis. Further, none of the 33 mothers from this study smoked during pregnancy.

Biological Pathway analysis

Following statistical testing, the study focused on biological pathways to explain the function of the identified CpGs. Function enrichment analyses were conducted using the genes of the discovered CpGs provided in the methylation label file (Infinium MethylationEPIC v1.0 B4 Manifest File). To identify genes linked to the CpGs for which the manifest did not provide any associated gene name, we used SNIPPER (<https://csg.sph.umich.edu/boehnke/snipper/>) [20] and the University of California Santa Cruz (UCSC) Genome Browser (<https://genome.ucsc.edu/>) [21]. The chromosome number and map info of the CpGs were queried (using Human GRCh37/hg19) and the nearest genes (maximum distance=250000 SNPs) to the site of the CpG were selected (up to two genes). Once all gene names were obtained, the full list was entered into Toppfun (<https://toppgene.cchmc.org/>) [22] to identify biological pathways related to these genes. Significant pathways adjusted for multiple testing (false discovery rate p-value <0.05) are presented.

Results

Study characteristics

From the total 542 mother-newborn dyads of IOW, 122 were included in the study. There was no significant difference between characteristics of the population analyzed and the whole F2-IOW cohort subjects (Table 1). Birthweight was normally distributed ranging from 1850 to 4450 grams. Among the 122 newborns, 20 and 19 had birthweights less than 3000 grams and more than 4000 grams, respectively.

The sub-sample from the Born Into Life cohort consisted of 33 mother-newborn dyads. There was no significant difference between the population analyzed and the whole Born into Life cohort in terms of characteristics (Table 1). The average birthweight was 3525 grams.

EWAS (IOW cohort)

The screening for identifying CpG sites with statistically significant differential DNAm related to birthweight using training and testing datasets yielded 283 CpGs, eight of which had a selection probability of 80% or higher (Table 2). These eight CpGs remained significantly related to birthweight after adjusting for newborn's gender, maternal peripheral blood cell types, maternal BMI, smoking during pregnancy, socioeconomic status, and multiple births. Among the eight CpGs, two were negatively and six were positively associated with offspring birthweight, respectively (figure 1). Structural equation modeling showed maternal DNAm to affect birthweight both directly and indirectly through gestational age in all candidate CpGs except for cg23153661 and cg 27394038. The latter two CpGs only directly affected birthweight and did not show any significant indirect effect on birthweight through gestational age (supplementary figure 1).

In addition, there was no correlation between maternal and cord blood DNAm levels of candidate CpGs, except for cg10715265, associated with DAB1 gene ($r = -0.23$, $p\text{-value} = 0.01$). Cord blood DNAm levels of the eight candidate CpGs were not associated with birthweight.

The DNAm levels of three CpGs were correlated in early and late pregnancy (cg00249511, $r_s = 0.25$, $p = 0.04$; cg04255048, $r_s = 0.28$, $p = 0.02$; cg23153661, $r_s = 0.29$, $p = 0.02$). Comparing the trend of DNAm at the eight candidate CpGs from age 18 to early and to late pregnancy using linear mixed models revealed no time-effect after cell-type adjustment.

Replication (Born-Into-Life cohort)

The directions of the estimates (association of maternal DNAm with offspring birthweight) of the candidate CpGs replicated in the Born Into Life cohort were concordant with those in the IOW cohort, except for one CPG (cg27239144). Of the eight candidate CpGs, three were marginally significant ($p \leq 0.20$, $n=33$) in the replication step (cg01816814, cg23153661, and cg17722033 with p -values= 0.06, 0.175, and 0.166, respectively).

Biological pathway analysis

The eight CpGs identified in the discovery phase were located on or close to 11 genes. Using ToppFun, biological processes related to cellular transport (import into cell, endocytosis, receptor mediated endocytosis), membrane invagination, and forebrain development were identified. Functional analysis of the genes associated with the three CpGs that were marginally significant in the replication, showed enriched biological processes related to endocytosis, protein glycosylation in Golgi, lipoprotein lipase activity positive regulation, and dorsal/ventral axis specification (Table 3).

Discussion

In DNAm measured in maternal blood during gestation, we found eight statistically significant CpGs to be associated with offspring birthweight located on or close to 11 genes. The smaller sample size of the Born Into Life cohort limited the replication and only three CpGs were marginally significant, corresponding to four genes *LMF1*, *NECAP1*, *VAX2*, and *CD207*.

Biological pathway analysis of three of the four genes enriched processes of endocytosis, membrane invagination, and vesicle organization.

Endocytosis in pregnancy is related to two processes, namely, maternal immune regulation and autophagy. Dendritic cells in uterine mucosa express C-type lectin receptors (CLRs) such as

Langerin (encoded by *CD207*), binding to cell surface carbohydrates, mediating their uptake via endocytosis in the first step of antigen presentation to the immune system.[23-25] CLRs, In addition to function as pathogen recognition receptors, mediate identification of carbohydrate structures on self-glycoproteins promoting self-antigens tolerance.[26] Dendritic cells of the uterine mucosa have been implied in pregnancy maintenance by establishing maternal immune tolerance of fetal tissues.[27] Achieving immune tolerance towards the fetus is required for creating a favorable maternal-fetal interface serving the growth and development of the fetus.[28]

Endocytosis is also involved in autophagy, a lysosome-mediated process maintaining cellular homeostasis by degrading useless or destructive intracellular materials.[29, 30] Autophagy has been implicated in uterovascular changes in pregnancy such as implantation and placentation.[31] Autophagy exists in endometrial stromal cells and epithelial cells and helps constitution of fetal-maternal interface by maintaining cellular homeostasis under physiological or pathological stress. [32] By sustaining a stable fetal-maternal interface, autophagy may be involved in fetal growth. In fact, increased autophagy have been reported in placentas obtained from pregnancies complicated by intra-uterine growth retardation.[30]

In addition to biological pathway analysis of gene clusters, we found potential linkages between three individual genes and pregnancy and fetal growth. The CpG cg01816814 associated with *LMF1* gene nearly gained statistical significance in the replication (P value=0.06). *LMF1* is an endoplasmic reticulum chaperone required for the post-translational activation of vascular lipases including lipoprotein lipase (LPL), hepatic lipase, and endothelial lipase.[33] The implication of maternal *LMF1* gene for offspring birthweight can be explained by its crucial role in regulation of maternal plasma lipids which are required substrates for fetal growth. [34] In the third

trimester, the activity of maternal adipose tissue lipoprotein lipase decreases and maternal fat depots break down as a result of increased lipolysis and lipid mobilization.[35] This change in maternal LPL activity leads to physiological maternal hypertriglyceridemia in late pregnancy.[35, 36] Maternal supply of triglycerides have been shown to be correlated with fetal lipid levels and fetal growth.[37] Triglycerides do not cross the placenta; however, placental LPL and endothelial lipase hydrolyze them, liberating fatty acids that can be taken up by the placenta and used by the fetus to grow.[38] Previous studies have reported a positive association between placental LPL enzymatic activity [37] and DNAm of placental *LPL* gene [39] with birthweight and neonatal adiposity.

SCYL2 gene, in addition to playing a role in positive regulation of endocytosis, is involved in Wnt signaling pathway. Different Wnt signaling components and ligands have been identified in the uterus promoting endometrial changes such as decidualization and endometrial gland formation. [40, 41] Ineffective decidualization have been reported in pregnancies complicated with intrauterine growth retardation. [42]

SCT gene codes for Secretin, a peptide hormone. Murine studies have shown secretin expression by uterine stromal cells from early pregnancy.[43] Secretin levels increases significantly in late pregnancy with highest level at 36 weeks.[44] Our data showed a positive correlation between DNAm of CpG cg00249511, associated with *SCT* gene, in early and late pregnancy, possibly implying the importance of secretin throughout gestation. Secretin and secretin receptor axis leads to the activation of intracellular secondary messenger system of cAMP, a strong inducer of decidualization.[44] The role of maternal secretin in decidualization could explain its association with fetal development and offspring birthweight. Moreover, secretin has lipolytic effects and is

involved in appetite regulation and glucose/insulin homeostasis [45], potentially influencing maternal metabolism and fetal development during pregnancy.

In addition to the maternal effects, several roles of secretin in fetal development, particularly in the developing brain, have been reported.[46] Placenta provides secretin to the developing fetus before it gains the ability to produce its own. [45] Interestingly, neonatal methylation levels of the corresponding receptor gene of *SCT*, namely, *SCTR* gene, have been associated with birthweight. [5]

Although correlated, birthweight and gestational age may present different processes.

Birthweight is determined by both the duration of gestation and fetal growth rate.[47] Using structural equation modeling, we observed direct and indirect effects of maternal DNAm at six candidate CpGs (cg01816814, cg04255048, cg10715265, cg00249511, cg27239144, and cg17722033) on birthweight through gestational age. This finding suggests that these CpGs influence birthweight by affecting both the duration of gestation and the fetal growth rate.

However, the other two CpGs, cg23153661 and cg 27394038 associated with *VAX2*, *CD207*, and *SFRS3*, showed no significant indirect effect on birthweight through gestational age.

Given that the methylation data of the candidate CpGs did not vary significantly with age (18 years, early, and late pregnancy), it is possible that the DNAm is driven by maternal genetic polymorphisms (methylation quantitative trait loci or methQTLs).[48] Hence, there is a need in future studies to assess whether maternal genetic polymorphisms of the linked genes are associated with birthweight of the child.

Our study has some limitations. First, we only evaluated DNAm extracted from maternal peripheral blood samples. Given the substantial differences in the epigenetic markings of distinct

cell types, CpG sites other than what we identified may be associated with offspring birthweight in other organs. Second, even though all maternal blood samples were collected in late pregnancy, they were not drawn at the same gestational week. Nonetheless, the gestational week at which the maternal blood was drawn had no association with the DNAm levels of the candidate CpGs after adjusting for blood cell types. Third, although directions of each association among CpGs and birthweight was established, we cannot state that the direction corresponds to gene expression. Lastly, our results were only marginally replicated, presumably due to the small sample size of the Born Into Life cohort. However, for three genes *LMFI*, *SCYL2*, and *SCT*, prior genetic and metabolic findings also support our results. Future studies with larger sample sizes are needed to evaluate all associations described in this study.

Conclusion

Our study (n=122) found eight maternal CpGs differentially methylated in association with offspring birthweight, three of them were replicated with marginal statistical significance in a small sample (n=33). The associated genes were linked to metabolic pathways involved in providing substrates for the developing fetus or in cellular processes such as endocytosis, possibly playing a role in sustaining a sufficient maternal-fetal interface. Overall, our findings contribute to the concept of maternal involvement in offspring birthweight. Additional studies are needed to elucidate the role of maternal DNAm in pregnancy and its association with offspring birthweight and other pregnancy outcomes. This can contribute to the identification of preventive or therapeutic targets for neonatal low and high birthweights. Furthermore, it is necessary to determine which environmental or maternal factors influence the differential maternal DNAm in order to identify targets for interventions reducing the risk of low or high birthweights. ~~Furthermore, measuring DNAm levels of maternal CpGs may have diagnostic~~

potential, recognizing mothers at risk for pregnancies complicated by non-optimal birthweight.

Finally, measuring DNAm levels of maternal CpGs may have a diagnostic potential recognizing mothers at risk for pregnancies ending with altered birthweights.

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Figure captions:

Fig.1 DNA methylation levels of statistically significant maternal candidate CpGs (β values) ~~and~~ in association with offspring birthweight (grams).

Legend: Solid lines and dashed lines represent regression lines and 95% confidence intervalCI, respectively.

Supplementary fig1 Structural equation modeling graphs showing the total effect of maternal DNAm on birthweight, indirect effect of maternal DNAm on birthweight through gestational age, and the direct effects between maternal DNAm, gestational age, and birthweight. Models are adjusted for newborn's gender, maternal peripheral blood cell types, maternal BMI, smoking during pregnancy, and socioeconomic status. Results are shown as beta values*100 (percentage)

*0.001 \leq P-value < 0.05, **P-value < 0.001