

Full Length Article

DNA methylation patterns in T lymphocytes are generally stable in human pregnancies but CD3 methylation is associated with perinatal psychiatric symptoms

Thalia K. Robakis^{a,*}, Seonjoo Lee^b, Elizabeth Werner^b, Grace Liu^b, Melissa Miller^c, Dennis Wylie^c, Frances A. Champagne^c, Martha Salas^d, Catherine Do^d, Benjamin Tycko^d, Catherine Monk^b

^a Icahn School of Medicine at Mount Sinai, New York, NY, 10029, USA

^b Columbia University Department of Psychiatry, New York, NY, 10032, USA

^c University of Texas at Austin Department of Psychology, Austin, TX, 78712, USA

^d Hackensack Meridian Health Center for Discovery and Innovation, Nutley, NJ, 07110, USA

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ABSTRACT

Objectives: To determine whether DNA methylation patterns in genes coding for selected T-lymphocyte proteins are associated with perinatal psychiatric distress or with complications of pregnancy.

Methods: T lymphocyte DNA was obtained from pregnant women across three time points in pregnancy and the postpartum period and epigenetic patterns were assessed using Illumina 450 K Methylation Beadchips. Seven selected genes critical for T cell function were analyzed for methylation changes during pregnancy and for associations of methylation patterns with psychiatric distress or with pregnancy complications, with particular attention paid to spatial aggregations of methyl groups, termed 'hotspots,' within the selected genes.

Results: In the candidate gene approach, DNA methylation density within a single cluster of 9 contiguous CpG loci within the *CD3* gene was found to be strongly associated with anxiety and depression in mid- and late pregnancy, and weakly associated with the presence of complications of pregnancy. Average DNA methylation density across each of the seven genes examined, and assay-wide, was found to be relatively stable across pregnancy and postpartum, but methylation within the *CD3* hotspot was more malleable and changes over time were coordinated across the nine cytosines in the hotspot. *CD3* CpGs did not pass array-wide tests for significance, but CpG clusters in two other genes, *DTNBP1* and *OXSRI*, showed array-wide significant associations with anxiety.

Conclusions: Despite the need for tolerating the fetal hemi-allograft, overall DNA methylation patterns in T lymphocytes are generally stable over the mid to late course of human pregnancies and postpartum. However, site-specific changes in DNA methylation density in *CD3* appear linked to both symptoms of depression and anxiety in pregnancy and, less strongly, to adverse pregnancy outcomes.

1. Introduction

Immune function in pregnancy is of critical importance for maternal and infant health, and is complicated by the requirement of tolerance for the immunologically foreign fetus. In addition, maternal psychological factors such as stress and anxiety could potentially alter immune function. In fact, among non-pregnant populations, it is well known that psychosocial factors including emotions, personality, social status, and psychiatric symptoms can adversely affect immune regulation with

significant health consequences (Lasselin et al., 2016). Epigenetic patterning in T lymphocytes might therefore be affected by maternal psychosocial factors in human pregnancy.

However, data on the possible role of psychiatric symptoms in the variation of pregnant women's immune activation, and the subsequent impact on health, remain limited (Christian, 2012; Osborne and Monk, 2013; Robakis et al., 2017). Given the substantial changes in immune function that accompany pregnancy, it is unclear to what degree findings from the general population will translate to pregnant women. Moreover,

* Corresponding author. 401 Quarry Road, MC, 5723, USA.

E-mail address: thalia.robakis@mssm.edu (T.K. Robakis).

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the genetic and epigenetic mechanisms by which psychosocial factors may be related to immune activity and gestational health outcomes have rarely been studied.

It has been established that maternal prenatal depression and anxiety are associated with complications of pregnancy and adverse neonatal outcomes. For example, neonates exposed to antenatal maternal depression are at greater risk for preterm birth (Eastwood et al., 2017; Grigoriadis et al., 2013). Reduced birth weight is another frequent finding (Grigoriadis et al., 2013), although this may be population-dependent as increases in birth weight have also been reported (Ecklund-Flores et al., 2017). Maternal antenatal anxiety also is related to preterm birth, perhaps more so than maternal depression (Rose et al., 2016; Pesonen et al., 2016; Ding et al., 2014). Antenatal depression also has been positively correlated with an increased risk for pre-eclampsia (Hu et al., 2015), a complication of pregnancy affecting about 5% of women in the United States. At least three longitudinal studies have identified pre-existing depression as a risk factor for the development of gestational diabetes, another common pregnancy complication (Bowers et al., 2013; Morrison et al., 2015), although not all investigators have obtained the same result (Ertel et al., 2014), and the question of the causal direction of this association remains open.

Maternal immune function also has been demonstrated to be of major importance to maternal and infant health during pregnancy. Three major categories of adverse pregnancy course are associated with immune dysregulation: preterm birth (Wei et al., 2010), pre-eclampsia (Ahn et al., 2011), and gestational diabetes (Wolf et al., 2004; Bo et al., 2005; Kinalski et al., 2005; Lowe et al., 2010). Given the significant overlap between the adverse perinatal outcomes independently associated with maternal psychiatric symptoms and maternal immune function, it has been hypothesized that alterations in immune function might mediate this association (Osborne and Monk, 2013; Leff-Gelman et al., 2016).

However, specific data to support this overarching hypothesis remain limited. Until recently, most of the available supporting data rested on demonstrations of elevated serum cytokine levels or immune cell populations in distressed pregnant women. Pregnant women with symptoms of depression have been shown to manifest elevated baseline serum proinflammatory cytokines IL-6, TNF- α , and IL-1 β (Christian et al., 2009; Cassidy-Bushrow et al., 2012) as well as an exaggerated immune response to vaccine challenge found in some (Christian et al., 2010) but not all (Blackmore et al., 2011; Walsh et al., 2016) studies. Negative emotions also have been correlated with higher levels of the immune cytokines IL-6 and IL-8 in both maternal and neonatal blood samples (Fransson et al., 2012). A longitudinal study of 23 cytokines in 51 women also recorded increases in IL-6 with increasing depressive symptoms, although overall reported no clear pro- or anti-inflammatory shift, and that immune profiles were better distinguished by demographic than by psychiatric variables (Osborne et al., 2019). Several other groups have published similar results, though primarily with very small sample sizes and highly heterogeneous methodologies (Osborne and Monk, 2013; Robakis et al., 2017). A somewhat larger study (N = 139) found that higher prenatal maternal depressive and anxious symptoms were positively correlated with serum levels of both Th1 and Th2 cytokines (Karlsson et al., 2017).

On a cellular population level, elevated lymphoproliferative responses to immune challenge in addition to higher levels of the immune cytokines IL-6 and IL-10 also have been documented in pregnant women with depressive symptoms (Mattes et al., 2009). Antenatal perceived stress has been associated with increased frequencies of regulatory T cells (Treg, crucial regulators of maternal-fetal tolerance) and lower birth weights (Ludwigs et al., 2013).

However, serum levels of cytokines are prone to rapid fluctuations and can be difficult to associate reliably with psychosocial or behavioral measures of ambient stress. Populations of immune effector cells are somewhat more stable over time, though also prone to alterations from upstream inputs. Epigenetic modifications to the genes coding for immune factors could represent an upstream factor in a causal chain from

maternal psychiatric distress to immune cell population and effector production, and ultimately, to gestational health and pregnancy outcomes. Among such modifications, CpG methylation is known to be propagated during cell divisions and thus could represent a more stable and reliable measure of maternal prenatal immune dysregulation relevant to maternal and infant health.

We hypothesized that a pathway from antenatal maternal psychiatric symptoms to altered immune function, and ultimately to poor pregnancy health and adverse outcomes, would proceed in part via specific epigenetic modifications of genes coding for T-lymphocyte associated proteins. Specifically, based on sample of women with blood draws at two time points in pregnancy and the postpartum as well as medical information on pregnancy course and birth outcomes, we aimed to determine whether women's prenatal psychiatric distress would be associated with DNA methylation of key effectors of immune function in maternal T lymphocytes. Further, we aimed to identify whether this variation in DNA methylation related to pregnancy complications and birth outcomes. We focused on genes for factors associated with mature T-helper (CD4) and cytotoxic (CD8) T cells (*CD4*); activated T lymphocytes (*CD3*); and regulatory T cells (*FOXP3*). We also assessed DNA methylation patterns in the *IL6* and *IL10* genes, which as noted above have been implicated in maternal stress by assays of circulating cytokines. We included a comparison candidate gene, *IGFBP1*, where DNA methylation has been associated with antenatal maternal stress (Mueller and Bale, 2008) but no specific connection to immune function is predicted.

Hypothesis. We hypothesized that pregnant women with high levels of depression, anxiety, and perceived stress in mid-pregnancy would show increased DNA methylation density at clusters of CpG dinucleotides within one or more of six candidate immunoregulatory genes (*FOXP3*, *CD3*, *CD4*, *CD8*, *IL6*, *IL10*). We further hypothesized that the methylation patterns that are associated with antenatal depression, anxiety, and perceived stress also would be positively correlated with increased rates of adverse maternal and infant pregnancy outcomes. In an additional exploratory analysis, we tracked the stability of these methylation patterns over the latter half of pregnancy and the postpartum period.

2. Methods

2.1. Participants

Healthy pregnant women, ages 18–45 were recruited through the Department of Obstetrics and Gynecology at Columbia University Medical Center (CUMC) as part of a larger study on maternal prenatal distress and fetal outcomes (Monk et al., 2016). Women were excluded if they acknowledged smoking or use of recreational drugs, lacked fluency in English or were multiparous or taking medications. Over a four-year period (2011–2015), 163 participants were recruited. This report describes results obtained from 58 participants from whom T cell data were available. All enrolled participants provided written-informed consent, and all procedures were approved by the Institutional Review Board of the New York State Psychiatric Institute.

2.2. Study procedures

Participants completed questionnaires at four timepoints: 9–12 weeks gestation (T1), 24–27 weeks gestation (T2), 34–37 weeks gestation (T3), and 4 months postpartum (PP). Blood samples for T cell analysis were obtained at 24–27 weeks gestation, at 34–37 weeks gestation, and at 4 months postpartum. Only a subset of participants (N = 12) provided blood samples at three time points. See study flowchart (Supplementary Figure 1) for summary.

2.3. Maternal characteristics and birth outcomes

Complications of pregnancy (N = 21, see Table 2), complications of

delivery (N = 11), postnatal complications (N = 4), gestational age at birth, head circumference, birth weight, C-section status, and sex of the infant, were determined from the medical records. Gestational age at study sessions was determined based on ultrasound examinations and last reported menstrual cycle. Maternal body mass index (BMI) was calculated using pre-pregnancy weight from self-report and measured height, along with maternal age.

2.4. Maternal psychiatric symptoms

Participants completed the self-reported Perceived Stress Scale (PSS (Cohen et al., 1983)) and two interview-based rating scales for psychiatric symptoms: the Hamilton Rating Scale for Depression (Ham-D (Williams, 1988)) and the Hamilton Anxiety Rating Scale (Ham-A (Hamilton, 1959)).

2.5. Analysis of CpG methylation in T cells

For collection of T lymphocytes, total CD3-positive T cells were prepared from the blood samples by negative selection using RosetteSep kits (Sigma). Genomic DNA was then prepared by standard SDS/proteinase K lysis followed by phenol-chloroform extraction and ethanol precipitation. The preparations were checked for quality (high molecular weight DNA) on ethidium-stained agarose gels. DNA, 500 ng, was bisulfite converted and analyzed according to the manufacturer's instructions for Illumina HumanMethylation 450 K BeadChips, with all assays performed at the Roswell Park Cancer Institute Genomics Shared Resource. Data were processed using Genome Studio software, which calculates the fractional methylation (AVG_Beta) at each CpG, after background correction and normalization to internal controls. All probes querying CpGs that overlapped common simple nucleotide polymorphisms (SNPs with minor allele frequency >1% in dbSNP build 138) were removed. In view of expected variations in CD3 T cell purity and percentages of other blood cell types, we performed deconvolution of the proportions of cell populations, namely CD4 T cells, CD8 T cells, natural killer, monocyte, granulocyte, and B cells, using a Minfi-based approach of the Houseman algorithm (Houseman et al., 2012) with the purified blood cell data contained in the Bioconductor package FlowSorted.Blood.450 k as a reference set (Reinius et al., 2012). Two samples were discarded for insufficient DNA yield; for the remainder, mean T cell fraction was 93% (range 66–100%). Percentage of T cells in each sample and CD4/CD8 ratio were then included as a variable in downstream analyses, as specified below (see Results).

2.6. Data analysis plan

Pearson correlations were calculated between psychiatric data (HAM-A, HAM-D, PSS) and obstetrical outcome data (birth weight, head circumference, gestational age at delivery), and point-biserial correlations between psychiatric data and the binary presence or absence of complications of pregnancy, delivery, and the postpartum period respectively.

39 women provided T cell samples at the second trimester timepoint (Table 1); one was removed from the analysis due to failure of T cell purification. Spearman's rank order correlation analyses were used to examine associations between DNA methylation outcomes and scalar psychiatric and obstetrical variables (HAM-A, HAM-D, PSS, gestational age, birth weight) at the 24–27 week time point, and to identify bivariate relations between differentially methylated CpG sites within *CD3*, *CD4*, *CD8*, *FOXP3*, *IL6*, *IL10*, and *IGFBP1* and clinical variables. T tests were used for binary variables (presence or absence of antenatal, perinatal, or postnatal complications). Spearman correlations were calculated between clinical scales (HAM-A, HAM-D, PSS) and DNA methylation density at each individual CpG locus in the dataset (97 loci after removal of cross-reactive probes) for the data from the 24–27 week time point (N = 38). Because of the high likelihood for chance associations to occur at

Table 1
Sample demographics.

Demographic Table	n = 39		n = 58	
	Mean (SD) or N (%)	Range	Mean (SD) or N (%)	Range
Maternal age (years)	31.33 (5.84)	20.26–44.66	30.77 (6.25)	20.26–44.66
Maternal Education (years)	15.74 (2.78)	10.00–23.00	15.43 (2.88)	10.00–23.00
Ethnicity				
Hispanic/Latina	21 (53.85)		34 (58.62)	
Race				
American Indian/Alaskan	14 (35.90)		20 (34.48)	
Asian	1 (2.56)		2 (3.45)	
Black/African American	6 (15.38)		8 (13.79)	
White/Caucasian	18 (46.15)		26 (44.83)	
Biracial	0		2 (3.45)	
Household Income				
\$0–\$15,000	2 (5.13)		3 (5.17)	
\$16,000–\$25,000	7 (17.95)		10 (17.24)	
\$26,000–\$50,000	8 (20.51)		14 (24.14)	
\$51,000–\$100,000	11 (28.21)		15 (25.86)	
\$101,000–\$250,000	10 (25.64)		14 (24.14)	
Above \$250,000	1 (2.56)		2 (3.45)	
Gestational Age at Birth (Weeks)	39.20 (1.28)	34.86–41.00	39.35 (1.22)	34.86–41.00
Birth Weight (Grams)	3369.49 (502.74)	2320–4470	3344.14 (461.51)	2320–4470
Baby Sex (female)	23 (58.97)		35 (60.34)	

individual loci given the size of the dataset, analysis focused on regions where multiple spatially contiguous loci were associated with a given clinical variable. This focus is based on a large body of research that documents spatial clustering among functionally associated CpGs (Suderman et al., 2012), and is in accordance with the generally accepted recognition that some of the primary functions of DNA methylation, such as the blocking of access to the transcription machinery, depend on the accumulation of multiple methyl groups over a single stretch of DNA (Boyes and Bird, 1992; Hsieh, 1994), thus contributing to the clustered patterns of DNA methylation that are observed over the genome.

Regions of DNA that included 5 or more spatially contiguous CpG loci where DNA methylation density was significantly ($p < 0.05$) associated with a variable of interest were termed 'hotspots' and were carried forward to the longitudinal analysis.

To access the significance of the identified hotspots, random permutation tests were conducted: we generated $B = 100000$ random permutation samples by permuting subject IDs in DNA methylation data, but preserving correlation within DNA methylation data and clinical outcomes. After computing Spearman's correlation and applying a 5% threshold, the empirical p-values were computed as the number of samples identifying those exact hotspots out of B random permutation samples.

After identifying hotspots, we conducted a mediation analysis to test whether the T cell DNA methylation explains the effect of maternal mood during pregnancy on the birth outcomes GA at birth, birth weight, and perinatal and postnatal complications. For the analysis, mediation R-package (v.4.4.2) (Tingley et al., 2014) was used and the indirect effect's 95% confidence intervals and p-values were derived with 5000 bootstrapping.

Spearman's rank order correlations were used as above to examine associations between DNA methylation outcomes at CpGs within these

Table 2
 Pearson and point-biserial correlations among psychiatric, obstetric, fetal, and neonatal outcomes. Maternal anxiety, depression, and perceived stress are associated with increased birth weight, greater head circumference, and increased incidence of complications of pregnancy. N = 58 (all subjects with methylation data; all subjects not represented at all time points).

	HAMD, T1	PSS, T1	HAMA, T2	HAMA, T3	HAMA, T3	HAMD, T3	PSS, T3	HAMA, PP	HAMD, PP	PSS, PP	Gestational Age	BW % for GA	Head Circumf	Antenatal Comp	Perinatal Comp	Postnatal Comp
HAM Anxiety, T1	0.838 ^a	0.404 ^b	0.555 ^a	0.316	0.439 ^b	0.16	0.018	0.221	0.221	-0.301	0.153	0.186	0.145	0.423 ^b	-0.258	-0.234
HAM	1	0.545 ^a	0.664 ^a	0.449 ^b	0.615 ^b	0.482 ^b	0.214	0.391	0.391	-0.13	0.286	0.345	0.224	0.503 ^a	-0.228	-0.168
Depression, T1		1	0.334	0.086	0.33	0.455 ^b	0.213	0.498	0.498	-0.008	0.341	0.225	0.053	0.211	0.009	0.205
Perceived Stress, T1			1	0.627 ^a	0.33	0.455 ^b	0.213	0.498	0.498	-0.008	0.341	0.225	0.053	0.211	0.009	0.205
HAM Anxiety, T2			1	0.710 ^a	0.688 ^a	0.458 ^a	0.621 ^a	0.699 ^a	0.699 ^a	0.226	0.295 ^b	0.309 ^b	0.415 ^a	0.441 ^a	-0.13	0.065
HAM			1	0.662 ^a	0.702 ^a	0.440 ^a	0.486 ^b	0.626 ^a	0.626 ^a	0.172	0.345 ^a	0.352 ^a	0.408 ^a	0.472 ^a	-0.061	0.043
Depression, T2				1	0.538 ^a	0.547 ^a	0.650 ^a	0.763 ^a	0.763 ^a	0.253	0.320 ^b	0.306 ^b	0.256	0.466 ^a	0.102	-0.011
Perceived Stress, T2				1	0.538 ^a	0.547 ^a	0.650 ^a	0.763 ^a	0.763 ^a	0.253	0.320 ^b	0.306 ^b	0.256	0.466 ^a	0.102	-0.011
HAM Anxiety, T3				1	0.782 ^a	0.493 ^a	0.514 ^b	0.510 ^b	0.510 ^b	0.1	0.21	0.208	0.27	0.346 ^b	0.078	-0.105
HAM				1	0.782 ^a	0.493 ^a	0.514 ^b	0.510 ^b	0.510 ^b	0.1	0.21	0.208	0.27	0.346 ^b	0.078	-0.105
Depression, T3					1	0.570 ^a	0.348	0.389	0.389	0.128	0.162	0.186	0.234	0.319 ^b	0.113	-0.098
Perceived Stress, T3					1	0.570 ^a	0.348	0.389	0.389	0.128	0.162	0.186	0.234	0.319 ^b	0.113	-0.098
HAM Anxiety, PP						1	0.478 ^b	0.565 ^a	0.565 ^a	0.228	0.181	0.235	0.136	0.494 ^a	0.299 ^b	-0.134
HAM						1	0.478 ^b	0.565 ^a	0.565 ^a	0.228	0.181	0.235	0.136	0.494 ^a	0.299 ^b	-0.134
Depression, PP							1	0.816 ^b	0.816 ^b	0.34	0.165	0.133	0.569 ^a	0.437 ^b	-0.117	-0.032
Perceived Stress, PP							1	0.816 ^b	0.816 ^b	0.34	0.165	0.133	0.569 ^a	0.437 ^b	-0.117	-0.032
Gestational Age								1	0.598 ^a	0.598 ^a	0.399 ^b	0.387	0.626 ^a	0.469 ^b	-0.128	-0.061
BW percentile for GA								1	0.598 ^a	0.598 ^a	0.399 ^b	0.387	0.626 ^a	0.469 ^b	-0.128	-0.061
Head									1	0.171	0.257	0.257	0.045	0.21	0.007	-0.018
Circumference										1	0.171	0.257	0.045	0.21	0.007	-0.018
Antenatal Complications											1	0.950 ^a	0.560 ^a	0.004	0.048	0.074
Perinatal Complications												1	0.546 ^a	0.11	0.046	0.035
Postnatal Complications																1

^a Correlation is significant at the 0.01 level (2-tailed).

^b Correlation is significant at the 0.05 level (2-tailed).

regions of interest and covariates of interest at the 34–37 week and postnatal time points, using the larger group of women (N = 58) who had each provided T cell data for at least two of three time points.

For the smaller group (N = 12) that had T cell DNA methylation data available at all three time points, trajectories of DNA methylation density over time were plotted (Fig. 4), and correlation coefficients for DNA methylation density measurements between 24–27 and 34–37 weeks, and between 34 and 37 weeks and postpartum, were calculated to quantify stability of methylation density over time (Supplementary Table 3).

We conducted array-wide analyses for the same sets of main hypotheses conducted as a supplement to the targeted gene approach. The multiple comparison correction was performed using the bump hunting method implemented in the bump hunter R-package (version 1.28.0) (Jaffe et al., 2012) (Aryee et al., 2014).

3. Results

3.1. Demographic, clinical, and newborn characteristics

Demographic, clinical, and birth outcome data for the 58 women providing data across the study, and separately for the 39 women who provided data at the 24–27 week time point, are summarized in Table 1.

3.2. Associations between maternal distress and neonatal outcomes

Pearson correlations between maternal distress at three time points and clinical variables of interest are shown in Table 2. Measures of maternal distress were relatively stable over time. Hamilton-Depression at T1 was highly correlated ($p < 0.01$) with Hamilton-Depression at both T2 and T3. Hamilton-Anxiety and Perceived Stress showed more loss of fidelity over time, with $p < 0.01$ for the repeated-measure correlations between the T1 and T2 values, but $p < 0.05$ for the repeated-measure correlations between the T1 and T3 values. See Supplementary Table 1 for changes in these parameters over the course of pregnancy.

Anxiety, depression, and perceived stress at 24–27 weeks were all associated with increased birth weight, increased head circumference, and greater gestational age at delivery. Rates of antenatal complication were significantly associated with distress measures at all time points. We also had data on maternal serum levels of IL-6 at T2 and T3; maternal serum IL-6 was not associated with any of the measures of distress or with complications of pregnancy or delivery at either time point (data not shown).

Subcategories of pregnancy complication contained too few subjects for meaningful analysis, however see Table 3 for a listing of all observed antenatal complications (N = 21). Eleven women had complications of delivery and four women had postpartum complications. Six women experienced both an antenatal complication and a complication of delivery. No woman experienced both a delivery complication and a postnatal complication. One individual experienced both an antenatal complication and a postnatal complication.

3.3. Associations between DNA methylation density in selected immune effector genes in T lymphocytes collected at 24–27 weeks with 24–27 week maternal distress and complications of pregnancy

DNA methylation density at numerous scattered individual CpG loci showed positive associations with maternal distress and complications of pregnancy, as expected for this dataset with many hypothesis tests. This analysis revealed one region of interest in which nine spatially contiguous CpG dinucleotides showed positive associations with second-trimester HAM-A and HAM-D. See Fig. 1 for heat map of all analyzed CpG loci in CD3. This region of DNA covered the 3' portion of the gene coding for the CD3-D chain, the 5' portion of the gene coding for the CD3-G chain, and the short intergenic region between them (Fig. 2). A schematic diagram showing the strength of associations at each of the

Table 3

Complications of pregnancy experienced by subject population. 21 of 58 participants experienced some complication of pregnancy. Numbers sum to greater than 21 due to comorbidity of several of the listed complications in the same individuals.

Complications of Pregnancy	Subjects Affected
Weight/metabolic	
Excessive weight gain	1
Obesity	7
Diabetes mellitus	1
History of polycystic ovarian syndrome	1
Vascular/hypertensive/pre-eclamptic	
Vascular complications	2
Pre-eclampsia/hypertension	2
Protein S Deficiency	1
Immunological	
History of hypothyroidism	1
Thyroidectomy	1
Infectious	
Yeast infection/UTI	2
Infection (unknown)	1
Other	
Progesterone therapy	1
History of osteosarcoma, history of respiratory distress from chemotherapy	1
History of abdominal myomectomy	1
History of subchorionic hematoma, resolved	1
Asthma	2
History of mental illness	1
Ovarian torsion	1
History of congenital heart defect, surgically treated	1
Anemia	1

individual loci comprising this region of interest is shown in Fig. 3. No regions of interest were identified for birth weight, gestational age at delivery, or perinatal or postnatal complications.

The likelihood of obtaining 9 consecutive CpG sites by chance was calculated as above to be 0.0096 for the association between DNA methylation density and HAM-D at 24–27 weeks. The association between mean DNA methylation density over the hotspot and HAM-D at 24–27 weeks remained significant after controlling for T cell fraction and CD4/CD8 ratio among the samples (initial standardized beta 0.333 and $p = 0.047$, standardized beta after control for T cell fraction and CD4/CD8 ratio 0.320 and $p = 0.030$). Importantly, using the results of our cell type deconvolution analysis, we additionally found that the CD4/CD8 ratio did not differ significantly between the during-pregnancy samples and post-pregnancy samples.

Mediation analysis showed that the association between second-trimester HAM-D and occurrence of complications of pregnancy was not mediated by DNA methylation of the CD3 hotspot, regardless of whether % T cell purity and CD4/CD8 ratio were controlled (with controls, 95% CI -0.03 to 0.04). The same results were found for gestational age at delivery (95% CI -15.35 to 7.56) and birth weight (95% CI -0.07 to 0.18).

3.4. General stability of methylation patterns during pregnancy

Data from the subset of women who provided T cell samples at each of three time points were used to map methylation density over time for each individual. Methylation density within most of the studied regions was stable over time, suggesting that epigenetic changes in the 7 candidate genes in T lymphocytes do not play a major role in the altered immune state of pregnancy (Supplementary Table 2). Moreover, when we performed a genome-wide analysis using the 450 K methylation data, comparing all during-pregnancy to all post-pregnancy samples and requiring a greater than 10 percent change in methylation with a false-discovery rate $FDR < 0.05$, we found no individual CpGs with significantly altered methylation.

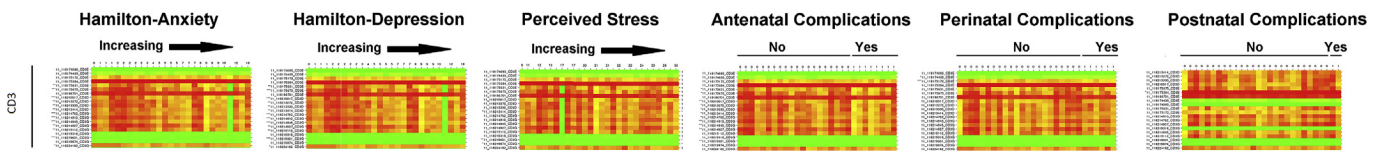


Fig. 1. Heat maps showing DNA methylation density at 97 measured loci at the 24–27 week time point, against Hamilton-Anxiety, Hamilton-Depression, Perceived Stress, and complications of pregnancy, delivery, and the postpartum period, for 38 individuals. Scores on clinical scales are sorted from lowest (least distress) to highest (most distress). Pregnancy complications are binary: no complication (sorted to the left) or some complication (sorted to the right). Green indicates greater methylation density, red indicates lower methylation density. Loci where methylation density is correlated with the given clinical scale are marked with asterisks (* = $p < 0.1$, ** = $p < 0.05$, *** = $p < 0.01$). A band of loci within CD3 shows greater methylation density associated with higher scores on the Hamilton-Depression scale. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

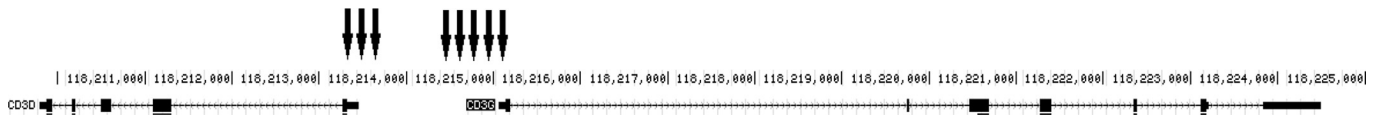


Fig. 2. Gene map showing the location of the methylation hotspot in the 3' region of CD3-D and 5'UTR of CD3-G. (hg19 assembly, chr 11, figure courtesy of UCSC genome browser.) Each arrow indicates an individual CpG locus where methylation density is positively associated with anxiety, depression, and antenatal complications of pregnancy (not to scale).



Fig. 3. Schematic illustration of strength of association for methylation density at individual CpG loci within CD3 with selected clinical variables. Top row: methylation data obtained at 24–27 weeks. Second row: methylation data obtained at 34–37 weeks. Columns: left to right, distress data obtained at 24–27 weeks, at 34–37 weeks, and at 4 months postpartum (Spearman correlation). Last column shows complications of pregnancy, delivery, and postpartum (T test). A ‘hotspot’ of 9 contiguous CpG loci within CD3 shows positive associations of DNA methylation density with psychiatric distress. Second trimester distress is more strongly associated than third trimester distress with hotspot methylation across pregnancy. Antenatal distress does not correlate with hotspot methylation density at 4 months postpartum. Hotspot methylation density in the third trimester is also associated with complications of pregnancy.

3.5. Associations of CD3 gene methylation with maternal symptoms and pregnancy complications

However, DNA methylation density within the CD3 hotspot was unusually variable across pregnancy and postpartum (Fig. 4A). Further, the absolute value of methylation density across the nine cytosines in the hotspot was quite tightly correlated, and directional changes in methylation density were synchronized across cytosines within an individual participant (see Fig. 4A), suggesting a coordinated function for DNA methylation at these nine adjacent sites.

Data from the subset of women who provided T cell samples for at least two time points per individual (N = 58) were used to investigate the temporal changes in the associations between methylation density at the CD3 region of interest and maternal symptoms over time (Fig. 3). Maternal psychiatric symptom data were available for the second and third trimester time points; data were not complete for the postpartum time point so this time point was omitted from the analysis.

Depression alone at the 24–27 week time point was associated with contemporaneous DNA methylation density at the CD3 region of interest (Fig. 3, top row, left column), and this remained robust to control for T cell purity and subfraction as above. However, depression and anxiety at 24–27 weeks were even more strongly associated with CD3 hotspot methylation density at 34–37 weeks (Fig. 3, top row, second column).

(T2 depression/T3 methylation: initial standardized beta = 0.497, $p = 0.007$; after control for CD4/CD8 ratio and T cell fraction, standardized beta = 0.529, $p = 0.005$. T2 anxiety/T3 methylation: initial standardized beta = 0.412, $p = 0.03$; after control for CD4/CD8 ratio and T cell fraction, standardized beta = 0.449, $p = 0.02$.)

Contemporaneous associations among distress variables at 34–37 weeks and hotspot-specific DNA methylation density at 34–37 weeks were much weaker than those between 24 and 27 week distress and 34–37 week methylation (Fig. 3, bottom row, second column), suggesting a forward causation from earlier distress to later DNA methylation. None of the measures of distress in pregnancy were associated with DNA methylation density in the postpartum period. Complications of pregnancy were weakly associated with DNA methylation density at the CD3 hotspot at 34–37 weeks, but not with DNA methylation density at 24–27 weeks.

3.6. Array-wide analysis of DNA methylation and maternal distress variables

In addition to our chosen candidate-gene approach, we also conducted an unbiased array-wide analysis of DNA methylation density with respect to scores on the anxiety, depression, and perceived stress measures. In an array-wide analysis using single CpGs without the hotspot

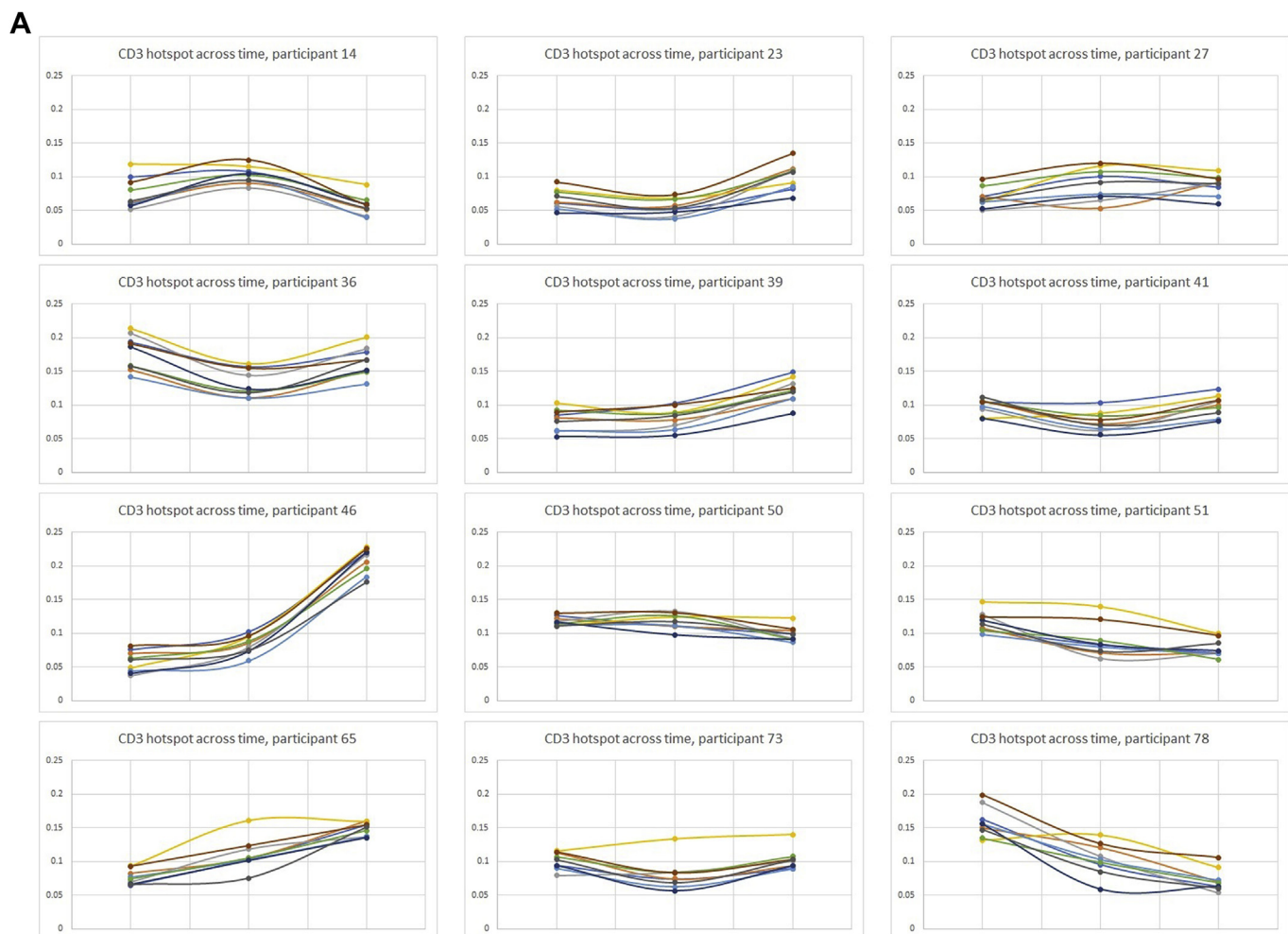


Fig. 4. DNA methylation density at 24–27 weeks, 34–37 weeks, and 4 months postpartum for 12 individual participants, for cytosines in CD3 located within the hotspot (Fig. 4A) or outside it (Fig. 4B). Methylation changes at hotspot CpGs appear coordinated, while methylation outside the hotspot is more stable and, when altered, changes independently of neighboring CpGs. (A). 9 individual CpGs within the CD3 hotspot. (B). Six individual CpGs outside the CD3 hotspot, each with mean methylation density in similar range to hotspot CpGs. Five CpGs with mean methylation densities above 0.25 were omitted from figure, but showed similar temporal stability.

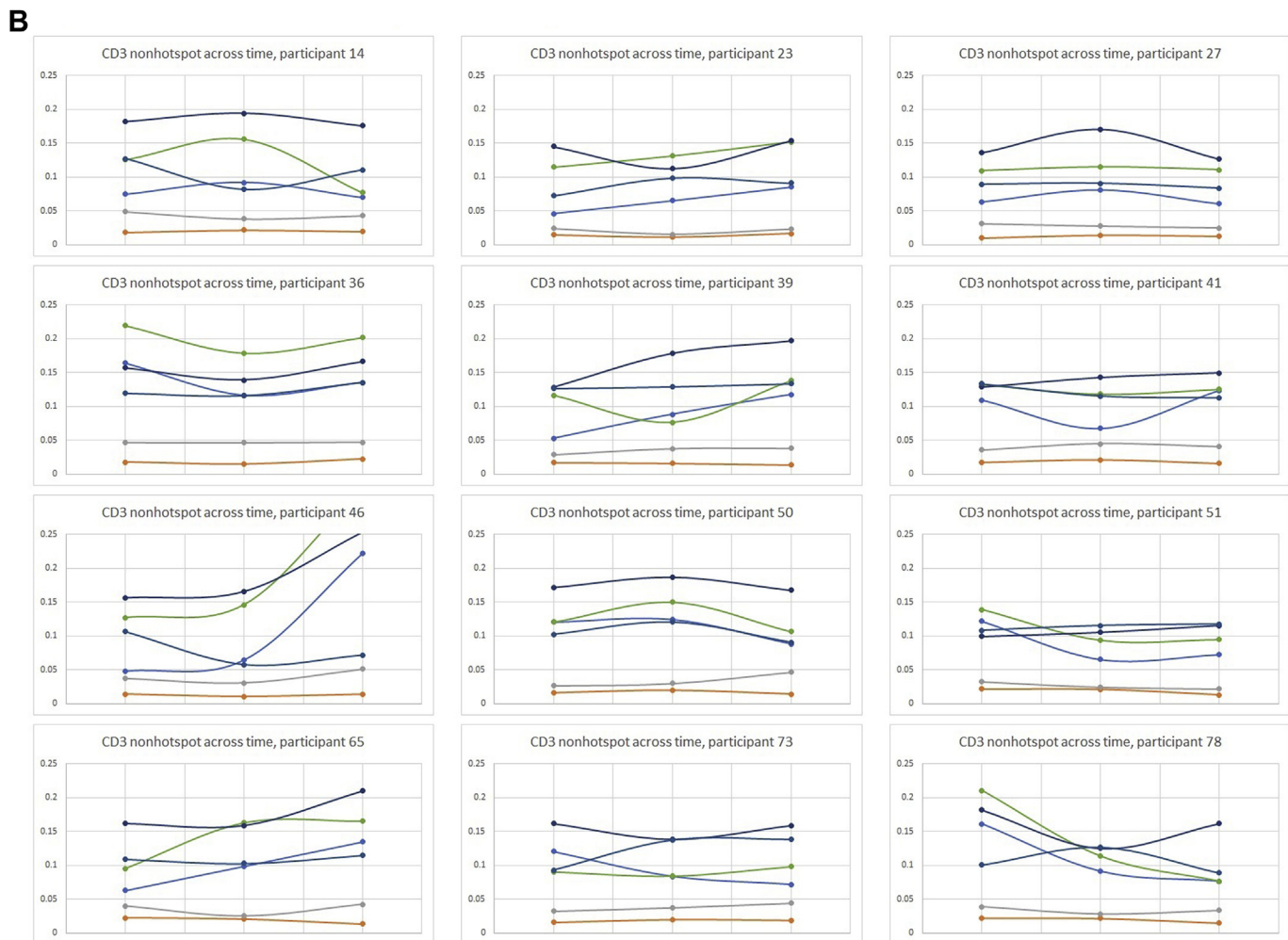


Fig. 4. (continued).

analysis, no results survived correction for multiple testing. A second analysis using the *bumphunter* method (Jaffe et al., 2012), a method for large-scale analysis that is comparable to our hotspot approach for individual candidate genes, yielded two results that passed statistical correction. A cluster of 15 CpG sites within *DTNBP1* was associated with scores on the anxiety and depression measures, and a cluster of 8 CpG sites within *OXSRI* was associated with scores on the anxiety measure. These two genes, which are known to be involved in neurocognition and cell membrane transport respectively, will be of interest for future epigenomic and biological studies.

4. Discussion

Maternal distress in pregnancy has previously been associated with adverse obstetrical outcomes; it has been hypothesized that this association could occur via excess immune activation occasioned by psychiatric symptoms and/or external stressors. However, a detailed understanding of the causal chain between maternal distress, immune activation, and adverse pregnancy outcomes has yet to emerge.

In this paper we show that maternal prenatal depression at 24–27 weeks of pregnancy is positively associated with DNA methylation density in a series of eight spatially contiguous CpG loci within the gene for the gamma and delta chain subunits of CD3, a co-receptor made up of multiple subunits that helps activate cytotoxic T cells and is expressed at all developmental stages of T lymphocytes in humans. We further provide exploratory results showing that multiple measures of distress at 24–27 weeks drive *CD3* DNA methylation at 34–37 weeks, and that

complications of pregnancy are moderately associated with *CD3* DNA methylation at 34–37 weeks. However, *CD3* DNA methylation did not mediate the association between psychiatric distress and complications of pregnancy. This result could be consistent with a mechanism in which *CD3* is only a small component of a larger network of genes whose coordinate regulation could mediate observed associations between distress and pregnancy complications.

4.1. Potential functional effects of altered *CD3* methylation

The CpG loci of interest were spread over a region that included the 3' region of *CD3D* and the 5' region of *CD3G*. The CD3 γ , δ , and ϵ subunits are highly homologous and can be incorporated alternatively to produce different subtypes of T cell receptor/CD3 coreceptor complexes (Kappes and Tonegawa, 1991), although the distinct functions of alternatively constituted coreceptor complexes are not yet well defined.

While we did not have any *in vitro* demonstration of the effect on gene expression, DNA methylation of 5' regions has been shown to result in reduced levels of gene expression in other contexts (Bell et al., 2011). In our work, this would be consistent with greater symptomatology leading to higher densities of *CD3G* DNA methylation and ultimately to lower expression of CD3 gamma chain. The role of DNA methylation in the 3' region is less well characterized, and it is possible that DNA methylation within the 3' coding region of *CD3D* mainly functions together with DNA methylation at more downstream portions of the region of interest to modulate transcription of *CD3G*.

It is thought that the gamma-subunits of CD3 are crucial for

maximizing antigen-triggered activation of the T cell receptor (Kim et al., 2010); thus it is possible that the downregulation of CD3 γ could possibly reduce the intensity of the response to antigen challenge. Alternatively, CD3 γ downregulation could be an epigenetic indicator of a more global reduction in CD3 $^+$ cells.

Downregulation of CD3 γ is not obviously supportive of an inflammation-based explanation of the connection between maternal symptoms and adverse obstetrical outcomes. However, it could be considered concordant with animal work showing overall downregulation of active T cell populations resulting from social stress in pregnant rats (Stefanski et al., 2005). Indeed, a mild downregulation of CD3 $^+$ cells has been documented as a normal development in pregnancy (Taylor et al., 2002), although this modest overall reduction hides the more functionally important shifts from Th1 to Th2 dominance and upregulation of Treg cells that support fetal tolerance in a healthy pregnancy (Polessa et al., 2014). A population of women with pre-eclampsia (Rein et al., 2002) were found to have a more marked downregulation of CD3 $^+$ cells than that observed in healthy pregnancy, indicating that an overall downregulation of T lymphocytes can be observed in disorders with known links to immune dysregulation.

Other inflammatory disorders of pregnancy have been associated instead with upregulation of active T cells. Stimulation by microorganisms linked to inflammation-driven preterm birth increases overall levels of activated T cells (both CD3 $^+$ CD4 $^+$ and CD3 $^+$ CD8 $^+$) (Friedland et al., 2016). Women with gestational diabetes were originally reported to have increased expression of CD3 δ (Lapolla et al., 2000), although in a larger sample this finding failed to replicate, with no separation from controls (Lapolla et al., 2005).

However, in general, changes in the numbers of all lymphocytes are likely far less informative than disease-specific alteration patterns in lymphocyte subpopulations. We did not find any methylation hotspots within the genes for *CD4*, *CD8*, or *FOXP3*, but were not able to examine the many other potentially relevant T cell subset markers.

Our analysis indicated that *CD3* methylation was not a significant mediator of the relationship between maternal depression and anxiety and pregnancy complications, suggesting that regulation of *CD3* is one link in a complex set of events that likely involves multiple parallel pathways.

There is an earlier published investigation of DNA methylation signatures of antenatal depression in peripheral T lymphocytes (Nemoda et al., 2015), which found no results of significance. However, these authors used a false discovery rate strategy to look over a much larger number of individual CpG loci. When repeating their approach, we also found no results of significance. It is likely that our restricted candidate-gene strategy revealed results that would not have passed the false discovery rate threshold with the larger number of hypothesis tests. Our strategy of identifying 'hotspots' where multiple contiguous CpG loci show associations of DNA methylation density with depressive symptoms takes advantage of the fact that physical clustering of methyl groups along a DNA strand is of known importance for affecting transcription efficiency (Deaton and Bird, 2011) to reduce the likelihood of false positive findings. Related methods have been described elsewhere for large datasets (Jaffe et al., 2012).

4.2. Delayed association between perceived stress in mid-pregnancy and *CD3* methylation in late pregnancy

A novel finding in our work was that anxiety, depression, and perceived stress levels at 24–27 weeks of pregnancy were associated more strongly with *CD3* DNA methylation density observed later, at 34–37 weeks of pregnancy, than with contemporaneous DNA methylation density.

The persistence and strengthening over time of DNA methylation patterns in *CD3* associated with psychiatric distress in earlier stages of pregnancy is clinically relevant because it suggests that from an immunological perspective, maternal psychiatric distress in mid-pregnancy is

potentially more deleterious than distress in the latter part. It is also consistent with suggestions from other investigations, for example Coussons-Read et al. (2007), who found that stress across pregnancy correlated with increased IL-1 β and IL-6 only in the 3rd trimester.

The temporal persistence of a DNA methylation pattern associated with mid-pregnancy psychiatric distress also informs our thinking on the causal directions of the associations between maternal distress, *CD3* methylation, and adverse pregnancy outcomes. We have suggested that distress in pregnancy contributes to risk for adverse pregnancy outcomes, in part via a mechanism involving *CD3* methylation. However, given only cross-sectional data, it could also be that women who already know they have complicated pregnancies are more likely to experience high levels of depression and anxiety. The association of the pattern of DNA methylation within *CD3* at 34–37 weeks with distress scores at 24–27 weeks suggests at least that DNA methylation follows, rather than precedes, the experience of stress.

It is important to note that there are likely cyclical relationships between some or all of these variables. Perceived stress or anxiety could potentially act via depression to affect DNA methylation density, which could be one explanation for the observed delay.

4.3. Stability versus variability of DNA methylation density across pregnancy and the postpartum period

For the majority of the CpG loci examined, DNA methylation density across time was highly stable, with Spearman coefficients between adjacent time points generally >0.95 and p-values generally <0.001. Within the *CD3* hotspot correlation coefficients were lower, and often nonsignificant. These results indicate that methylation density within the *CD3* hotspot is less stable across pregnancy than mean methylation density over time for the rest of the regions studied. This accords with our finding that methylation density within this hotspot can correlate with clinical measures of psychiatric distress, and suggests that while methylation density over the genome as a whole is likely to be highly stable across pregnancy, there may be subregions where methylation density is more malleable and can be affected by, or affect, events that occur during the pregnancy.

4.4. A note on depression, obesity, and birth weight

One unusual finding in our work is that more severe symptoms of depression were associated with higher birthweights. This is in contrast to the bulk of the published literature on this topic, although reported associations have been weak and not always consistent (Grigoriadis et al., 2013). On the other hand, this reverse association has also been found in other cohorts (Ecklund-Flores et al., 2017), and it is possible that the weak overall association of depression with lower birth weight found in the literature as a whole masks differential associations in particular subgroups of women. In particular, if depression is associated with greater insulin resistance and higher serum glucose in pregnancy, as has been theorized elsewhere (Robakis et al., 2017; Kozhimannil et al., 2009), it is possible that this could lead to an overall association of depressive symptoms with higher birth weights, particularly in populations predisposed to insulin resistance. It is notable that the most common antenatal complication in our sample was maternal obesity (N = 7). However, likely due to small sample size, we did not find statistically significant associations between maternal obesity and any of the psychiatric or obstetrical outcomes under study.

4.5. Tolerant of the fetal hemi-allograft in pregnancy is not accounted for by widespread alterations in epigenetic patterns in maternal T lymphocytes

A pertinent negative finding in our study is that no individual CpG dinucleotide assay-wide was found to change significantly in T lymphocytes from peripheral blood samples over the time course of human pregnancy. We suggest that this may be due to the tendency of CpG

methylation to function in groups or islands, such that changes in methylation density at one individual CpG are small and only become functionally relevant when yoked to those at surrounding CpGs. In this case, analytical tools that screen for highly significant alterations in methylation density at individual CpGs in the genome may not be correctly designed to identify the relevant changes. Rather, tools that isolate windows of coordinately methylated sites (Wang et al., 2015) may be needed to home in on epigenetic contributions to immune regulation in pregnancy.

4.6. Limitations and future studies

The most obvious limitation to our dataset is its small sample size of 58. Using our targeted candidate gene approach (not the entire set of 450 K CpG probes) for assessing correlations of methylation with maternal anxiety and depression, and requiring clusters of differentially methylated CpGs, both serve to mitigate the problem of statistical power being reduced by multiple comparisons. At the same time, the findings in our array-wide analysis suggest that follow-up studies on the DTNBP1 and OXSR1 genes may lead to further insights to epigenetic changes due to maternal anxiety and depression.

5. Conclusions

Overall this work provides support for the Hypothesis that maternal prenatal psychiatric symptoms are associated with changes in DNA methylation patterns in specific subregions of genes that are central to T lymphocyte function. This suggests that spatial specificity is an important element of the contribution of the epigenetic code to immune regulation in pregnancy. These findings contribute to our nascent understanding of the molecular pathways by which maternal psychiatric symptoms can affect immune function and ultimately mother and infant health. Further research is needed to identify additional factors of relevance and clarify the many intersecting and diverging pathways that can lead from maternal distress to adverse outcomes of pregnancy.

Declaration of competing interest

The authors report no financial relationships with commercial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbih.2020.100044>.

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