

Replication of Epigenetic Postpartum Depression Biomarkers and Variation with Hormone Levels

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DNA methylation variation at *HP1BP3* and *TTC9B* is modified by estrogen exposure in the rodent hippocampus and was previously shown to be prospectively predictive of postpartum depression (PPD) when modeled in antenatal blood. The objective of this study was to replicate the predictive efficacy of the previously established model in women with and without a previous psychiatric diagnosis and to understand the effects of changing hormone levels on PPD biomarker loci. Using a statistical model trained on DNA methylation data from $N=51$ high-risk women, we prospectively predicted PPD status in an independent $N=51$ women using first trimester antenatal gene expression levels of *HP1BP3* and *TTC9B*, with an area under the receiver operator characteristic curve (AUC) of 0.81 (95% CI: 0.69–0.92, $p < 5 \times 10^{-4}$). Modeling DNA methylation of these genes in $N=240$ women without a previous psychiatric diagnosis resulted in a cross-sectional prediction of PPD status with an AUC of 0.81 (95% CI: 0.68–0.93, $p = 0.01$). *TTC9B* and *HP1BP3* DNA methylation at early antenatal time points showed moderate evidence for association to the change in estradiol and allopregnanolone over the course of pregnancy, suggesting that epigenetic variation at these loci may be important for mediating hormonal sensitivity. In addition both loci showed PPD-specific trajectories with age, possibly mediated by age-associated hormonal changes. The data add to the growing body of evidence suggesting that PPD is mediated by differential gene expression and epigenetic sensitivity to pregnancy hormones and that modeling proxies of this sensitivity enable accurate prediction of PPD.

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INTRODUCTION

Postpartum depression (PPD) affects between 10 and 20% of women (Josefsson *et al*, 2001; Miller, 2002; Pearlstein *et al*, 2009) and has significant effects on both mother and child (Breese McCoy, 2011; Cuijpers *et al*, 2008; Field, 2011; Hirst and Moutier, 2010; O'Hara, 2009; Soufia *et al*, 2010). Certain populations have higher rates of PPD including 30% in women with a history of depression and 52% of women with bipolar disorder (Viguera *et al*, 2011). A growing body of evidence indicates that increased sensitivity to gonadal hormone levels such as estrogen mediate the biological vulnerability to PPD. Early work by Bloch *et al* (2000) provided clear evidence that women with a previous history of PPD are sensitive to drastic hormonal changes, whereas more recent studies have demonstrated significant

gene expression changes in estrogen-responsive genes of women at risk for PPD (Mehta *et al*, 2014). Work out of our laboratory also provided evidence for a PPD-specific increased sensitivity to estrogen on the epigenetic level (Guintivano *et al*, 2013a). We demonstrated that women at risk for PPD exhibit more DNA methylation changes at loci that are sensitive to epigenetic reprogramming in the mouse hippocampus, a brain region thought to be involved in mediating estrogen's effects on mood (Walf, 2010). From this data set, we identified two biomarker loci (*HP1BP3* and *TTC9B*) that were capable of prospectively predicting PPD in humans with an area under the receiver operator characteristic curve (AUC) of 82%. Importantly, DNA methylation of *HP1BP3* was associated with a number of inflammatory cell-type elevations occurring in assayed antenatally depressed relative to antenatally euthymic women (Guintivano *et al*, 2013a) and required incorporation of cellular heterogeneity metrics into the model to achieve an accurate prediction across both subgroups.

Bioinformatic analysis suggests that *HP1BP3* and *TTC9B* may be involved in mediating synaptic plasticity (Guintivano *et al*, 2013a), a key facet of the antidepressant properties of

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Table 1 PPD Biomarker Model Performance

All	PPD	non-PPD	AUC	95% CI	PPD Diagnosis p^a	HP1BP3 p^a	TTC9B p^a	Cell type p^a
<i>Prospective gene expression cohort</i>								
1st Trimester	29	22	0.81	0.69–0.92	$< 1 \times 10^{-4}$	0.036	$< 1 \times 10^{-4}$	0.66
3rd Trimester	33	28	0.64	0.5–0.78	0.073	0.392	0.073	0.83
<i>Antenatally euthymic</i>								
1st Trimester	15	22	0.83	0.69–0.96	0.002	0.049	0.002	0.83
3rd Trimester	15	28	0.66	0.49–0.83	0.098	0.021	0.098	0.81
<i>Antenatally depressed</i>								
1st Trimester	14	22	0.80	0.65–0.95	0.001	0.073	0.00099	0.33
3rd Trimester	18	28	0.63	0.46–0.79	0.18	0.85	0.18	0.90
Antenatally euthymic	HDRS ≥ 14	HDRS < 14	AUC	95% CI	PPD Diagnosis p^a	HP1BP3 p^a	TTC9B p^a	Cell type p^a
<i>FRAMES cohort</i>								
Postpartum	5	235	0.81	0.68–0.93	0.01	0.014	0.0099	NA

^aPermutation p -values based on 10 000 iterations.

estradiol in the hippocampus (ter Horst, 2010) and potentially of high relevance to susceptibility to PPD. We believe the predictive efficacy of the biomarker model lies in the ability of relative epigenetic differences in these estrogen-responsive genes to model PPD-associated sensitivity to estrogens. Of course, the estrogens are not the only reproductive hormones that undergo marked changes during pregnancy and the postpartum period. Progesterone and its metabolites also change considerably. Some studies in the past have indicated that progesterone withdrawal may be associated with PPD (Harris *et al*, 1994). Recent work has focused on allopregnanolone, one of progesterone's 3α -reduced metabolites and a strong allosteric modulator of the GABA-A receptor (Bali and Jaggi, 2014; Bristot *et al*, 2014; Schumacher *et al*, 2014). It is therefore also possible that our biomarker model may be associated with changes in progesterone or allopregnanolone.

The objectives of this study were to independently replicate our previously published PPD prediction model in women with and without a prior psychiatric history, to further investigate the DNA methylation status of PPD biomarker loci with changing pregnancy hormone levels and over periods of major hormonal change.

MATERIALS AND METHODS

Human Samples

Subjects derived from two prospective cohorts designed to study PPD and two cohorts where DNA was taken long after pregnancy. The prospective cohorts included the Johns Hopkins Prospective PPD sample reported on previously by our group (Guintivano *et al*, 2013a) and a Prospective Gene Expression PPD cohort generated by Mehta *et al* (2014), which is a study investigating gene expression in pregnant women prospectively followed until they do or do not develop PPD. Both prospective cohorts were performed in

women with previous diagnoses of mood disorder (Guintivano *et al*, 2013a; Mehta *et al*, 2014). The Franconian Maternal Health Evaluation Studies (FRAMES) cohort focused on psychiatrically healthy women with prospective evaluation of depression and blood drawn for DNA at 1–3 years postpartum. The Generations of Recurrent Early Onset Depression (GenRED) cohort involved retrospective symptom assessment and blood draws. Detailed information on study subjects is available below and in Table 1, Supplementary Table S1.

Johns Hopkins prospective PPD cohort. We recruited 93 pregnant women with a history of either Major Depression or Bipolar Disorder (I, II, or NOS) and prospectively followed them during pregnancy and after delivery to identify genetic and clinical characteristics that precede the development of a postpartum depressive episode. Approximately one-third of the sample had bipolar disorder. The average age of the participants was 30.6 ± 6.3 and 70% of the sample was Caucasian. Participants were managed by their treating psychiatrist as clinically indicated and were evaluated during each trimester of pregnancy and then 1 week, 1 month, and 3 months postpartum. Women were classified as being depressed if they met DSM-IV criteria for a major depressive episode (MDE) based on a psychiatric interview at each time point (first, second, and third trimester and 1 week and 1 month postpartum). Childhood sexual abuse status was binarily coded as a response to the question 'Were you sexually assaulted as a child?' during a clinical interview. Although serum hormone levels were obtained longitudinally from multiple time points, blood was drawn for DNA analysis at only one time point per individual in this cohort. The trimester of blood draw is depicted in Supplementary Table S1. Prospective human subjects research at Johns Hopkins was conducted under IRB protocol # 00008149.

Prospective gene expression PPD cohort. Data from this cohort was generated by Mehta *et al* (2014) and was downloaded from the Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE45603. Subjects reportedly derived from a longitudinal cohort recruited at the Emory Women's Mental Health Program. The sample was 33-year-old on average and ~85% Caucasian. Approximately 59.6 and 40.4% of the sample had a diagnosis of MDD and bipolar disorder, respectively, based on the Structured Clinical Interview for DSM-IV Axis I Disorders. Depressive symptoms were assessed in the first and third trimester using the Beck Depression Inventory, the 17-item Hamilton Depression Rating Scale (HDRS), and the Edinburgh Postnatal Depression Scale. Depression diagnoses in the first trimester and postpartum are based on the HDRS score >14, which represents the score on this scale demonstrated to be maximally predictive of experiencing a MDE in the postpartum period (Ji *et al*, 2011).

GenRED cohort. The retrospective sample derived from the GenRED cohort at Johns Hopkins consisting 84 women non-menopausal aged was 39.7 ± 0.093 years at the time of blood draw. Data derived from EBV transformed lymphoblasts from women who had had a pregnancy. The sample was 92% Caucasian, 1.2% African American, 1.2% Asian, and 3.6% unreported. The menstrual cycle status at the time of blood sampling was not recorded. The frequency of psychiatric diagnosis was 44.6% major depressive disorder (MDD), 2.4% bipolar disorder type II, and 49% with no diagnosis. PPD in this cohort consisted of women who retrospectively reported experiencing depression in the postpartum period. Women experiencing depression symptoms antenatally that became euthymic during the postpartum period were classified as controls. Details on antenatal and PPD frequency per cohort are displayed in Table 1. Collection of the GenRED sample was performed under IRB protocol # 00035775.

FRAMES cohort. DNA was obtained from a subset of 421 women enrolled in the FRAMES and who were evaluated for genetic associations with postpartum outcomes in previous studies (Mehta *et al*, 2012). Of these samples, a total of $N=240$ passed quality control following subsequent analyses. All women were Caucasian and the mean age of the sample was 32.7 ± 0.018 years. Women responded to the HDRS prospectively during the third trimester, between 48–72 h, and between 6–8 months after parturition and PPD was classified as scores >14. It has been demonstrated that a score of 14 or higher on the HDRS is ~80% sensitive and specific to identify the presence of a MDE as determined by SCID in both the early and late postpartum period (Ji *et al*, 2011). All blood drawn for this study ranged in age between 1–3 years after parturition. The menstrual cycle status at the time of blood sampling was not recorded. The study was approved by the Ethics Committee at Erlangen University Hospital, Nuremberg, Germany. All participants received detailed information and provided written consent. Human subjects research in this population at Johns Hopkins was performed under IRB protocol # 00049309.

Illumina HM450 Microarray Data

Genome-wide DNA methylation data generated on the Illumina Human Methylation 450 (HM450) bead array were generated in 51 women from the Women's Mood Disorder Prospective sample previously (Guintivano *et al*, 2013a). Data can be found on the GEO (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE44132. DNA methylation helps to define cell types and as such, variation in cellular composition in heterogeneous tissues such as blood has the potential to bias study results if not appropriately corrected for (Guintivano *et al*, 2013b). As previously published, cell sub-fraction percentages were quantified for CD8-T cells, CD4-T cells, B cells, monocytes, and granulocytes by inputting DNA methylation signatures of 473 loci into an algorithm designed for quantification of the cell types using DNA methylation proxies (Houseman *et al*, 2012).

Sodium Bisulfite Pyrosequencing

Bisulfite conversion was carried out using EZ DNA Methylation Gold Kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. Nested PCR amplifications were performed with a standard PCR protocol in 25 μ l volume reactions containing 3–4 μ l of sodium-bisulfite-treated DNA, 0.2 μ M primers, and master mix containing Taq DNA polymerase (Sigma Aldrich, St Louis, MO). Primer sequences can be found in Supplementary Table S2. PCR amplicons were processed for pyrosequencing analysis according to the manufacturer's standard protocol (QIAGEN, Germantown, MD) using a PyroMark MD system (QIAGEN) with Pyro Q-CpG 1.0.9 software (QIAGEN) for CpG methylation quantification.

Gene Expression Data

Gene expression data generated on the Illumina HumanHT-12 V4.0 expression beadchip by Mehta *et al* (2014) was downloaded from GEO accession GSE45603. Raw data were normalized with the variance stabilizing transformation method (Huber *et al*, 2002) using the 'justvsns' function from the vsn package in R. Relative gene expression values for *HP1BP3* and *TTC9B* were extracted from the normalized data set for subsequent analysis. We quantified the relative proportions of CD8-T, CD4-T, B cell, monocyte, and granulocyte proportions using the CellMix package in R (Gaujoux and Seoighe, 2013) based on reference data in 42 probes available in both GSE45603 and those available in the reference data set generated by Abbas *et al* (2009).

Hormone Data

Participant blood was collected at each visit in four 10 ml EDTA tubes and were immediately centrifuged at 4 °C for 30 min. The serum was then aliquoted into 2 ml microcentrifuge tubes, snap frozen on dry ice, and immediately stored in a –80 °C freezer. The blood samples we chose for analysis were from women who had data for at least one antepartum blood draw and one postpartum visit with psychological data. Blood was analyzed with the following kits: Allopregnanolone EIA kit from Arbor Assays LLC (Ann Arbor, MI, USA) Cat 3 KC44-H1, Progesterone EIA kit

from Alpco (Salem, NH, USA) catalog # PROHU-E01, Estradiol Alpco Elisa kit catalog number 11ESPHU-E 1.

Statistical Analysis

All statistical tests were performed in R (<http://www.r-project.org/>). Using an Anderson–Darling test from the *nortest* package, all distributions of data that rejected the null hypothesis of normality were subsequently evaluated with non-parametric tests. All statistical tests performed were two tailed and a $p < 0.05$ is considered significant. Unless otherwise specified \pm denotes the SEM.

PPD prediction for all cohorts was achieved using the previously published model (Guintivano *et al*, 2013a):

$$E(\text{PPD}_i) = a + b\text{HP1BP3} + c\text{Cell type}_j + d(\text{HP1BP3} \times \text{Cell type}_j) + e\text{TTC9B}$$

Where PPD status for individual (i) was modeled as a function of an interaction of *HP1BP3* DNA methylation with cell-type variable (j), where j is the ratio of monocyte to non-monocyte counts (See Method S1 for relevant rationale), controlling for additive covariate *TTC9B* DNA methylation. To apply our DNA methylation model to gene expression scores when applied to the Prospective Gene Expression cohort, we rebuilt the training set-derived model from the Johns Hopkins Prospective Cohort after first converting DNA methylation at *HP1BP3* and *TTC9B* to z-scores in order to standardize the variation between the two loci relative to each other (Supplementary Figure S1). For the FRAMES cohort, cell-type information was not available and was not controlled for. Similar to results reported by Mehta *et al* (2014), psychiatric medication status did not influence PPD prediction model performance in the Johns Hopkins Prospective PPD cohort and were not included in subsequent analyses (Supplementary Result S1).

Sliding window analysis was used in the investigation of age-related changes to DNA methylation, whereby DNA methylation values for each gene were averaged for all women within 5 years of age for each age between 25 and 50.

RESULTS

Independent Prospective Replication of our PPD Predictive Model in Women with Mood Disorder

We attempted to apply our PPD biomarker model to the Prospective Gene Expression cohort generated by Mehta *et al* (2014) across multiple time points during pregnancy. Input of gene expression z-scores generated for *HP1BP3* (ILMN_1701169) and *TTC9B* (ILMN_1682123) and the monocyte to non-monocyte ratio generated significant predictions for PPD from first trimester but not third trimester blood (Figure 1, Table 1). Incorporation of cell-type ratio into the model as an interacting factor was necessary for accurate PPD prediction across antenatally euthymic and depressed women (Supplementary Result S2, Supplementary Figure S2). The predictive accuracy from first trimester blood was similar ~80% when modeling only PPD cases deriving from antenatally euthymic or depressed women separately (Table 1), whereas third trimester-derived predictions were not significant (Table 1). Permutation analysis of each model

component separately was employed to understand model factors contributing to predictive efficacy. Both *TTC9B* and *HP1BP3* gene expression significantly contributed to model performance in the antenatally euthymic and combined sample for the first trimester time point.

Unlike our originally published study, no significant associations of the monocyte to non-monocyte ratio were observed with antenatal depression status; however, a non-significant decrease was observed at the first trimester time point (Wilcoxon Rank Sum Test, $\text{PPD} = 0.24 + 0.0067$, non- $\text{PPD} = 0.23 + 0.0021$, $p = 0.085$), suggesting that antenatal depression-associated variation in cell type may be driving the improved predictability of the model during this time point. Indeed, application of an alternative model trained on the Johns Hopkins Prospective cohort but lacking the interacting cell-type ratio covariate generates a lower AUC of 0.75 (95% CI: 0.6–0.88, Model $P = 0.001$, *HP1BP3* $P = 0.78$, *TTC9B* $P = 0.0099$) and permutation analysis reveals that the *HP1BP3* component is no longer significantly contributing to the predictive efficacy.

Independent Replication of PPD Predictive Model in Women with no Previous Psychiatric History

PPD model efficacy was assessed for the ability to detect women with HDRS scores above 14 at 6–8 month postpartum in a cohort of women with no previous history of psychiatric diagnosis. Women with high HDRS scores at 2–3 day postpartum were excluded, as high HDRS scores at this time may be associated with more standard pregnancy-related mood fluctuations. The PPD prediction model generated a significant AUC of 81% in this cohort (Figure 3a, Table 1). Similar to the other DNA methylation models, both *HP1BP3* and *TTC9B* significantly contributed to model performance (Table 1). Prediction accuracy in the FRAMES cohort increased in sensitivity, specificity, and significance with increasing severity of depression scores in the postpartum period (Figure 2b, Supplementary Table S3).

Age as a Macro Model of Hormone Withdrawal

We used the GenRED sample to assess the change in DNA methylation in PPD biomarker loci over time. Using a sliding window analysis, women with a history of PPD showed significantly anti-correlated trajectories to non-PPD women as a function of age for *HP1BP3* ($\text{Rho} = -0.5$, $p = 0.006$) and *TTC9B* ($\text{Rho} = -0.8$, $p = 1.8 \times 10^{-7}$) (Figure 3). The same sliding window analysis employed in the FRAMES cohort identified similar anti-correlated trajectories between PPD and non-PPD women for *HP1BP3* ($\text{Rho} = -0.65$, $p = 7.1 \times 10^{-4}$) and *TTC9B* ($\text{Rho} = -0.65$, $p = 7.8 \times 10^{-4}$) (Figure 3).

Association of PPD Biomarker Loci with Hormone Levels

HP1BP3 and *TTC9B* DNA methylation levels were assessed for association to serum hormone levels of estradiol, progesterone, and allopregnanolone during the second and third trimester in the Johns Hopkins Prospective cohort. No relationship was observed between the number of live births per subject and gonadal hormone levels or PPD biomarker

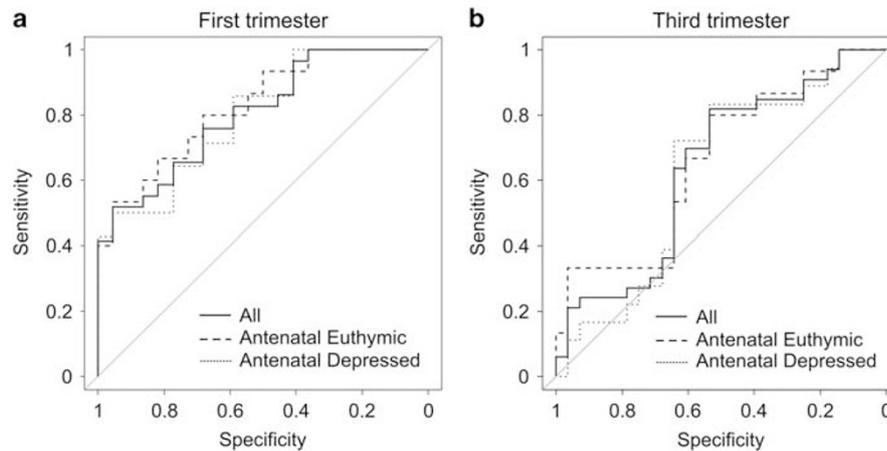


Figure 1 Receiver operator characteristic curves of prospective PPD prediction. Receiver Operator Characteristic (ROC) curves depicting the sensitivity (y axis) as a function of specificity (x axis) for prediction of PPD status in the Prospective Gene Expression cohort during the first (a) and third (b) trimesters.

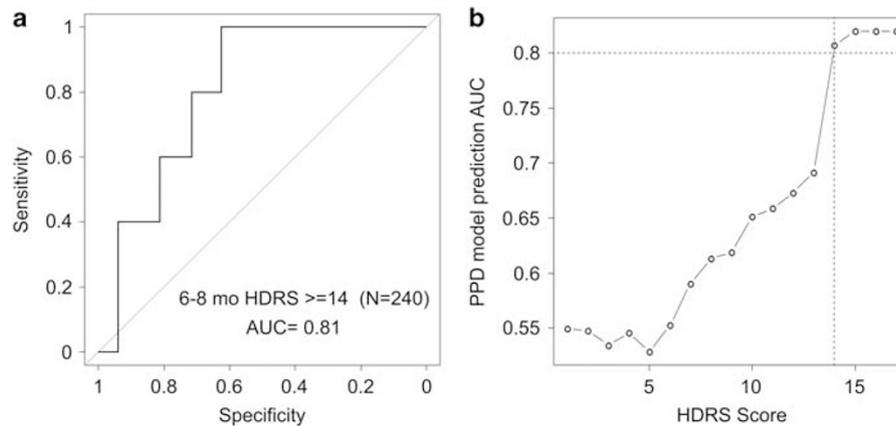


Figure 2 PPD prediction in women without a psychiatric history. (a) Receiver operator characteristic (ROC) curves depicting the sensitivity (y axis) as a function of specificity (x axis) for prediction of 6–8 months HDRS scores ≥ 14 status in the FRAMES cohort of women from the general population. (b) A plot of the performance of PPD model prediction as measured by AUC (y axis) as a function of the HDRS cutoff signifying affected individuals (x axis). The horizontal line denotes the threshold of a 'good' biomarker at an AUC of 0.80, whereas the vertical dashed line denotes an HDRS cutoff of 14.

loci (Supplementary Result S3). As hormone levels varied as a function of gestational age (Supplementary Figure S3), linear models adjusting for the change in gestational age per individual were employed to control for associations owing to variation in sampling time. No significant associations were observed between *HP1BP3* and any of the investigated hormones, whereas *TTC9B* DNA methylation was positively correlated with third trimester levels of estradiol ($\beta = 0.0058 \pm 0.0021$, $F = 4.8$, $df = 2/26$, $p = 0.01$) (Figure 4a). When we divided the cohort by depression status at the third trimester, this effect remained significant in women who were antenatally euthymic ($\beta = 0.0058 \pm 0.0026$, $F = 4.39$, $df = 2/16$, $p = 0.038$), but not in those who were antenatally depressed ($\beta = 0.0033 \pm 0.0045$, $F = 0.48$, $df = 2/7$, $p = 0.49$). In an effort to assess the directionality of the observed association, we assessed third trimester estradiol levels as a function of second trimester *TTC9B* DNA methylation and observed a non-significant positive association in antenatally euthymic women ($\beta = 0.0093 \pm 0.0042$, $F = 2.74$, $df = 2/7$, $p = 0.061$) that was markedly weakened when assessing the

combined sample of antenatally euthymic and depressed women together ($\beta = 0.0057 \pm 0.0037$, $F = 1.43$, $df = 2/11$, $p = 0.15$). We next assessed the change in hormone levels from second to third trimester time points to rule out an association with a pre-existing hormonal state. We observed a similar relationship of estradiol to *TTC9B* methylation in antenatally euthymic women when assessing the change in estradiol levels from second to third trimester ($\beta = 0.0087 \pm 0.0043$, $F = 2.13$, $df = 2/8$, $p = 0.078$) (Figure 4b) that was weakened by the addition of antenatally depressed subjects ($\beta = 0.0057 \pm 0.0036$, $F = 1.36$, $df = 2/12$, $p = 0.13$). Notably, second trimester estradiol levels were not associated with second trimester *TTC9B* DNA methylation ($\beta = 0.0005 \pm 0.0026$, $F = 0.039$, $df = 1/14$, $p = 0.85$).

In light of the observed moderate evidence that epigenetic variation at earlier time points may associate with the trajectory of hormonal changes during pregnancy, we re-evaluated *HP1BP3* DNA methylation levels in association with the change in hormone levels. Third trimester allopregnanolone was not associated with *HP1BP3* DNA

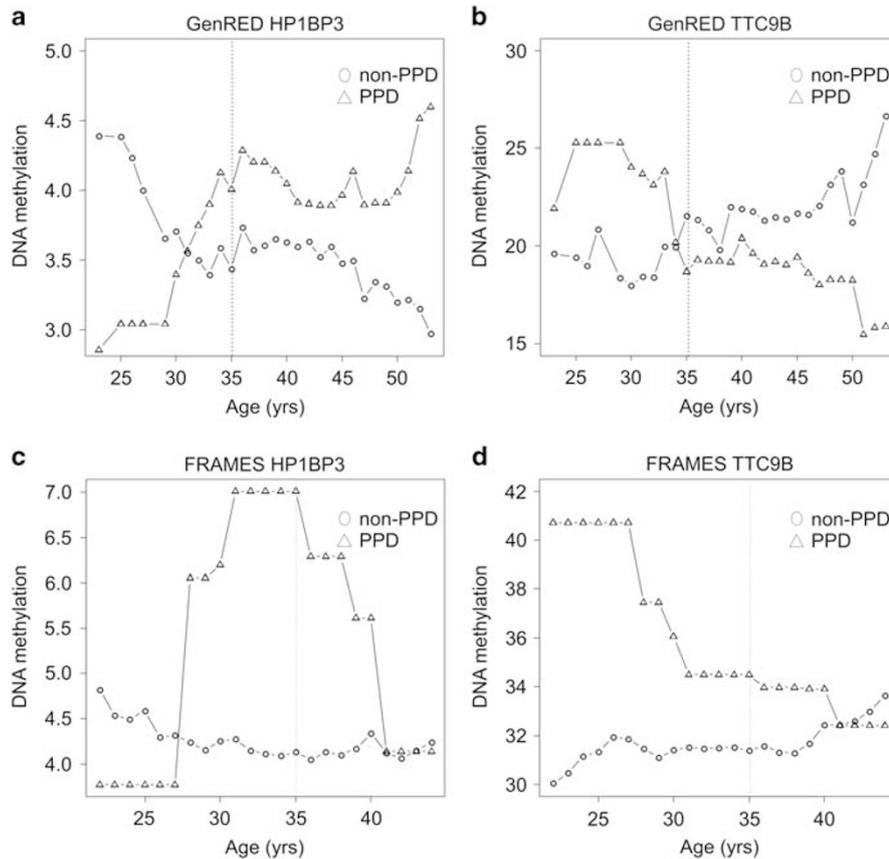


Figure 3 PPD biomarkers across the reproductive years. Plots of the mean DNA methylation (x axis) for PPD (triangles) and non-PPD (circles) cases for the *HP1BP3* (a) and *TTC9B* (b) genes over a 10-year sliding window as a function of age (y axis) in the GenRED cohort. Plots of the mean DNA methylation (x axis) for PPD (triangles) and non-PPD (circles) cases for the *HP1BP3* (c) and *TTC9B* (d) genes over a 10-year sliding window as a function of age (y axis) in the FRAMES cohort. The vertical dashed line represents an age of 35 years and denotes the approximate age in the female population when estrogen levels begin to decline.

methylation ($Rho = -0.17$, $p = 0.32$) (Figure 4c); however, as in the case with *TTC9B* DNA methylation and estradiol, second trimester *HP1BP3* DNA methylation was associated with third trimester allopregnanolone levels in antenatally euthymic women ($\beta = -0.36 \pm 0.12$, $F = 5.18$, $df = 2/5$, $p = 0.026$) but not in the combined sample ($\beta = -0.29 \pm 0.21$, $F = 1.94$, $df = 2/10$, $p = 0.18$). In addition, significant negative associations were observed for the change in allopregnanolone levels from second to third trimester in the antenatally euthymic ($\beta = -0.46 \pm 0.14$, $F = 8.35$, $df = 2/5$, $p = 0.022$) (Figure 4d) but not antenatally depressed samples ($\beta = -0.33 \pm 0.30$, $F = 1.85$, $df = 2/10$, $p = 0.3$). As was the case with our findings concerning *TTC9B* and estradiol, it is notable that the primary effect appears to be derived from the antenatally euthymic group (Supplementary Figure S4). To corroborate these findings, an exploratory analysis in the Prospective Gene Expression cohort found evidence for an overrepresentation of third trimester allopregnanolone associated loci with first trimester *HP1BP3* expression but not third trimester *HP1BP3* expression (Supplementary Result S4, Supplementary Figure S5A). Similarly, first trimester but not third trimester *TTC9B* gene expression was associated with third trimester estradiol-associated loci (Supplementary Result S4, Supplementary Figure S5B).

DISCUSSION

Using gene expression values in an independent prospective cohort of 51 women, our model prospectively predicted PPD with a similar accuracy to our original report at ~80%, suggesting that our model remains a valid predictor of future PPD risk. The success of the model in a gene expression sample despite being trained on a DNA methylation sample suggests that epigenetic variation at PPD biomarker loci is likely to be associated with expression. This supposition is corroborated by the observation of an association with monocyte to non-monocyte ratio with *HP1BP3* gene expression in a direction similar to that observed for DNA methylation in the original study. As the regions of relevant epigenetic change in *HP1BP3* and *TTC9B* reside within or proximal to the promoters for each gene, it is not unreasonable to assume that there may be a relationship to blood gene expression; however, this should be assessed in future studies as we lacked a data set to evaluate this specifically. Future studies should investigate if epigenetic variation at *HP1BP3* and *TTC9B* can be mediated by genetic variation as has been observed for other epigenetic associations with PPD (Bell *et al*, 2015).

The original report by Mehta *et al* did not incorporate a metric of cellular heterogeneity and was reportedly unable to

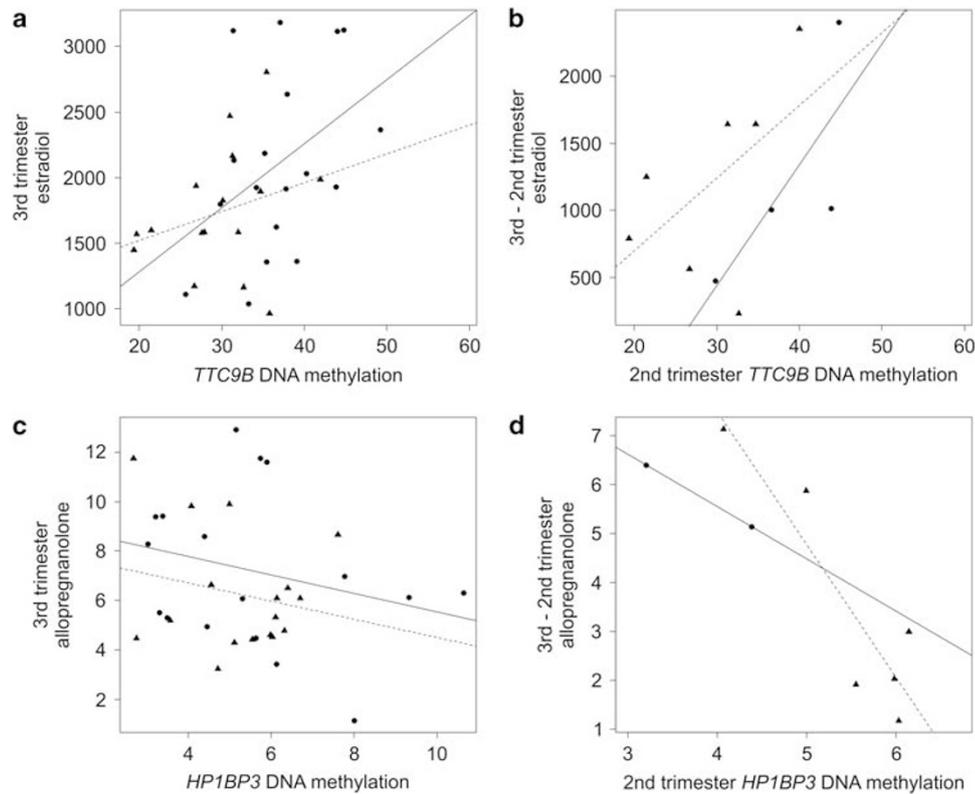


Figure 4 PPD biomarkers and pregnancy hormone trajectories. (a) A scatterplot of the third trimester estradiol levels in (y axis) as a function of *TTC9B* DNA methylation (x axis) for women who did (triangles, dashed regression line) and did not (circles, solid regression line) develop PPD. (b) A scatterplot of the change in estradiol from second to third trimester (y axis) as a function of second trimester *TTC9B* DNA methylation levels (x axis) for women who did (triangles, dashed regression line) and did not (circles, solid regression line) develop PPD. (c) A scatterplot of the third trimester allopregnanolone levels in (y axis) as a function of *HP1BP3* DNA methylation (x axis) for women who did (triangles, dashed regression line) and did not (circles, solid regression line) develop PPD. (d) A scatterplot of the change in allopregnanolone from second to third trimester (y axis) as a function of second trimester *HP1BP3* DNA methylation levels (x axis) for women who did (triangles, dashed regression line) and did not (circles, solid regression line) develop PPD. All data derives from the Johns Hopkins Prospective cohort.

predict PPD status in those women with antenatal depression, whereas our model appeared to function similarly in antenatally euthymic and depressed women, possibly owing to the incorporation of cell-type ratio information. Permutation analyses demonstrated that *HP1BP3* contributed significantly to the prediction of PPD in antenatally euthymic but not antenatally depressed women. We further demonstrated that application of an alternative model lacking the interacting cell-type ratio eliminated the ability of *HP1BP3* variation to contribute significantly to the predictive efficacy of our model. Taken together, the data corroborate the previously published data (Guintivano *et al*, 2013a), indicating that changes in cell-type ratio may confound the association of *HP1BP3* to PPD status and must be carefully controlled for in future studies. Unlike our original study, however, cellular heterogeneity demonstrated only moderate evidence for association with antenatal depression status in the gene expression cohort, suggesting that there are likely other factors not accounted for in our model that distinguish depression that continues into the postpartum period from depression arising only postpartum; these may be of critical importance for improving the predictive capacity of the model. In the Johns Hopkins Prospective cohort, direct assessment of hormone levels indicated that DNA methylation at earlier time points was

indicative of the levels of estradiol and allopregnanolone for *TTC9B* and *HP1BP3*, respectively, and these data were stronger in antenatally euthymic as compared with antenatally depressed women. Together these results suggest that women developing PPD who were euthymic during pregnancy may have unique hormonal milieu relative to those depressed during pregnancy.

Importantly, these prospectively assessed hormone associations argue for a contributing role of epigenetic variation to the etiology of varying hormone levels as opposed to being merely an effect of hormonal sensitivity; however, a robust replication of these findings in larger cohorts and functional studies are warranted to support this claim. Such a role may explain why the PPD prediction model generated by Mehta *et al* (2014), on this data set was most predictive during the third trimester, whereas our model performed best during the first trimester. Mehta *et al*'s model was enriched for estrogen receptor target genes, which may exhibit the most prominent differences during the third trimester when levels of pregnancy-associated estrogens are highest. Alternatively, *HP1BP3* and *TTC9B* variation at earlier time points may be more important not only for reflecting PPD-associated hormonal sensitivity but potentially for contributing to later time point hormonal outcomes. Notably, application of these two genes to PPD prediction in the originally published

DNA methylation sample demonstrated similar predictive efficacies independent of the antenatal time point at which blood was drawn (Guintivano *et al*, 2013a), suggesting the trimester-specific limitation on predictive efficacy may be specific to gene expression studies.

The observed associations of PPD biomarker loci with hormone changes corroborates previously reported bioinformatic evidence, suggesting both *TTC9B* and *HP1BP3* may be involved in modulating hippocampal synaptic plasticity (Guintivano *et al*, 2013a). Neither *TTC9B* nor *HP1BP3* have extensive evidence regarding their gene functions that may be of etiological relevance to PPD. Both genes have ties to estrogen signaling. *TTC9B* expression has been shown to be responsive to gonadal hormones (Cao *et al*, 2006) and is discussed in depth below. *HP1BP3* associates with ER β in culture (Nassa *et al*, 2011), binds to chromatin specifically at the repressive histone 3 lysine 9 trimethylation (H3K9me3) mark, and is expressed in most tissues with 10-fold higher levels in the brain (Garfinkel *et al*, 2015). Knockdown of *HP1BP3* by siRNA in culture leads to significant expression changes in hundreds of genes, suggesting it is involved in transcriptional regulation (Dutta *et al*, 2014; Garfinkel *et al*, 2015). Interestingly, some genes with the most significantly altered expression in culture experiments are known to mediate progesterone inhibition of estradiol signaling in the uterus, such as *MIG-6* (Yoo *et al*, 2015). Of those 12 loci consistently affected by *HP1BP3* knockdown in both of the above studies, three may have implications for mood. *ITGA6* is downregulated in a chronic mild stress-based depression paradigm in rodents (Orsetti *et al*, 2008), *EIF4B* is implicated in mTOR signaling of relevance for depression (Jernigan *et al*, 2011), and *GLO1* is implicated in BDNF signaling (Karpova *et al*, 2014), a downstream consequence of estrogen signaling in the hippocampus. Murine knockout of *HP1BP3* leads to growth retardation (Garfinkel *et al*, 2015), further suggesting a possible role for modulation of trophic factors in the context of the brain. Although the conclusions regarding brain-specific function of *HP1BP3* DNA methylation variation are limited at this time and must be interpreted with caution, the available data to date suggest a relationship with mood, mediated by either by downstream gene expression changes or neurosteroid levels, may be possible and warrants further study.

A contribution of *HP1BP3* variation to allopregnanolone levels is one way by which this gene may affect mood. The considerable animal research on the anxiolytic effects of allopregnanolone has been summarized by Schule *et al* (2014). Significant research links low levels of allopregnanolone to depressed mood (Backstrom *et al*, 2014; Eser *et al*, 2006; Le Melledo and Baker, 2004; Padberg *et al*, 2002; Pinna *et al*, 2006; Schule *et al*, 2014; Strohle *et al*, 1999; Uzunova *et al*, 2006) and other evidence links mood states or histories thereof with alterations in the ratio of allopregnanolone to its precursors (Girdler *et al*, 2012; Schiller *et al*, 2014). In the perinatal period specifically, Deligiannidis *et al* (2013), in a small sample, found no relationship between pregnancy allopregnanolone and the development of PPD, whereas Hellgren *et al* (2014) found significantly lower levels of allopregnanolone in depressed pregnant women when compared with healthy controls (Hellgren *et al*, 2014). In a separate analysis, our group found that low levels of allopregnanolone measured in the second trimester predicted

the development of PPD ($p = 0.01$); this effect was driven by women who were euthymic in the second trimester (Osborne *et al*, under review).

The effects of estradiol on hippocampally mediated mood are widely supported by estrogen receptor knockout experiments (Walf *et al*, 2008), 17 β -estradiol administration experiments (Osterlund *et al*, 2005), and selective ER antagonists and agonists (Walf and Frye, 2005). Furthermore, estradiol administration has been shown to increase synaptic plasticity and dendritic spine density within the hippocampus (ter Horst, 2010), whereas withdrawal from pregnancy levels of estradiol results in decreased hippocampal BDNF expression (Suda *et al*, 2008) and suppressed hippocampal neurogenesis (Green and Galea, 2008). Finally, high as compared with low E2 doses may inhibit neurogenesis in the dentate gyrus (Tanaka and Sokabe, 2013), suggesting that PPD-associated estrogen sensitivity may have deleterious consequences on mood in at-risk women. As such, *TTC9B* would be expected to have numerous consequences on mood and hippocampal synaptic plasticity through an alteration of estrogen trajectories.

Corroborating the assertion that *TTC9B* variation may contribute to estrogen sensitivity, a recent study identified that a close homolog of *TTC9B*, namely *TTC9A*, functions to regulate estrogen receptor alpha (ER α) levels (Shrestha *et al*, 2015). Importantly, *TTC9A* and *B* are highly conserved and share all regulatory protein domains (Shrestha *et al*, 2015), suggesting that they may have functional similarities such that *TTC9B* may contribute to the sensitivity of estrogen signaling implicated in PPD. An important possibility requiring further study is that these loci may be involved in feedback inhibition, as another study suggested that *TTC9A* appeared to be a target of the ER α in mammary tissue (Shrestha *et al*, 2012) and previous research out of our laboratory and others implicates that *in vitro* and *in vivo* estradiol treatment alters *TTC9B* expression (Cao *et al*, 2006) and methylation (Guintivano *et al*, 2013a), respectively.

A feedback regulation mechanism may reconcile the PPD-specific age-related trajectories of *TTC9B* and *HP1BP3* methylation with the above data, implicating a possible causal role for these loci in contributing to hormonal sensitivity. For example, using data generated in the GenRED and FRAMES cohorts, we demonstrated that the trajectory of DNA methylation for PPD biomarker loci appeared to be reflective of the average levels of the estrogens over a woman's life span and is significantly anti-correlated between PPD at risk and non-at risk women, further corroborating the assertion that their biomarker efficacy is based on their ability to be reflective of epigenetic sensitivity to estrogens. During the reproductive years, on average PPD biomarker loci appear to exhibit relative methylation differences similar to those observed in the antenatal period when estrogen levels are high, though they may vary with fluctuations as a result of the menstrual cycle not detectable in our data. However, as estrogen levels drop after age ~ 35 , relative DNA methylation levels at *HP1BP3* and *TTC9B* at risk women begin to diverge in the opposite direction between PPD and non-PPD and speaks to what may be observed for these loci in the postpartum period. An intriguing possibility warranting further study is that PPD biomarker epigenetic variation may be predictive of other female-specific mood disorders such as perimenopausal

depression. Three out of four studies have observed significant associations between postpartum mood symptoms and perimenopausal depressive symptoms (Payne *et al*, 2009). A caveat of this interpretation is that we did not measure estrogen levels with age and are basing this conclusion on the average levels of estrogen fluctuation in women. Other potential caveats are that PPD diagnoses in the GenRED samples are based on retrospective report; however, a replication of our observations in the FRAMES cohort where depression was derived prospectively supports the observations that differing epigenetic trajectories occur in women who will experience depression postpartum. Importantly, the data suggest that validation efforts of PPD biomarker model prediction may be possible in retrospective blood samples taken from women who have experienced PPD.

Such validation efforts did appear possible in the FRAMES cohort, where application of the PPD biomarker prediction model demonstrated a high ability to identify women exhibiting depressive symptoms at 6 months postpartum. We focused on this time period to avoid classifying incidences of 'baby blues' (Breese McCoy, 2011) as PPD. Critically, unlike our original study and the data set generated by Mehta *et al*, these results demonstrate that our model may be efficacious in a general population sample without a history of mood disorder. It is of note that only a small number (2.1%) of women developed depressive symptoms postpartum compared with the reported averages of 10–20% (Josefsson *et al*, 2001; Miller, 2002; Pearlstein *et al*, 2009). Permutation analyses increase the confidence that the high AUC values observed are not a result of small numbers of cases. Interestingly, the possible fluctuation of gonadal hormone levels as a function of normal menstrual cycling did not appear to affect our predictive accuracy. The reasons for this are unclear and may be related to either the low levels of normal cycling relative to pregnancy level hormones at the measured time point or the timing of epigenetic change in response to hormonal variation, both of which should be evaluated in future studies. Notably, accounting for cell-type variation was not necessary to enable accurate prediction in the FRAMES cohort, possibly because DNA was derived from blood taken long after pregnancy and was no longer influenced by inflammatory cell-type changes at the time of measurement.

In conclusion, our study provides two independent replications of the predictive efficacy of our PPD biomarker model in both a high risk and general population sample and provides additional evidence of the potential functional relevance of *HP1BP3* and *TTC9B* to disease etiology. In light of the devastating impact of PPD on both mothers and their children (Breese McCoy, 2011; Cuijpers *et al*, 2008; Field, 2011; Hirst and Moutier, 2010; O'Hara, 2009; Soufia *et al*, 2010), the epigenetic PPD biomarker may facilitate efforts by a growing number of states to implement early screening and intervention for PPD.

FUNDING AND DISCLOSURE

Drs Kaminsky and Payne are co-inventors listed on a patent application for DNA methylation at biomarker loci related to PPD. Dr Kaminsky is listed on patents for use of epigenetic information at the SKA2 locus to predict suicidal behavior

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