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Maternal night shift work is associated with stochastic epigenetic mutations (SEMs) in the placenta

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2018

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## Abstract

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By Jinze Li

Circadian disruption has been associated with many negative health outcomes, such as cognitive impairment, metabolic syndrome, psychiatric illness and also associated with changes in epigenetics, especially DNA methylation. But fewer studies have examined the effects of circadian disruption during pregnancy on maternal and child health. This research was based on 227 mother-infant pairs from the Rhode Island Child Health Study (RICHS). Among the 227 participants, there are 48 women who reported night shift work and 161 women did not. We quantified placental stochastic epigenetic mutations (SEMs) via DNA methylation which was measured with paired Illumina 450K and EPIC microarrays. Using linear regression and Poisson regression we tested the association between night shift work during pregnancy and SEM counts in the placenta. And we also performed enrichment analysis on the CpGs that had placental SEMs to know whether placental SEMs were enriched in particular biological pathways or were randomly distributed throughout the genome. We found that maternal night shift working had a positive and significant association with SEM counts without covariates ($\beta = 105.18$; $p=0.01$; 95%CI = (21.09, 189.28)) and with covariates, including maternal ethnicity, maternal gravita, delivery method, birthweight group, gender, maternal smoking status and adversity score ($\beta = 107.14$; $p=0.02$; 95%CI = (17.67, 196.60)) via linear regression. Again, with the Poisson regression model, maternal night shift working had a positive and significant association with SEM counts without covariates ($\beta = 0.23$; $p=0.01$; 95%CI = (0.05, 0.41)), and with covariates ($\beta = 0.24$; $p=0.01$; 95%CI = (0.05, 0.42)). We also found that SEMs were more likely to occur in genes that belong to “neuron projection morphogenesis”, “plasma membrane bounded cell projection morphogenesis”, “neuron differentiation”, “cell projection morphogenesis” and “neuron projection development”, “glycosphingolipid biosynthesis - globo and isoglobo series” and “nicotine addiction” pathways. In conclusion, mothers with night shift work tend to have more CpG sites with SEMs than those without nightshift work, with or without controlling for maternal ethnicity, maternal gravita, delivery method, birthweight group, gender, maternal smoking status and adversity score. This demonstrates that SEMs offer an innovative alternative approach to studying the impacts of environmental exposure on placental epigenetics.

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## Introduction

Circadian rhythm describes physiological or behavioral changes that follow 24-hour cycles which regulate the internal body systems to coordinate with changes in the external environment. The circadian system is related to nearly all physiology. Circadian disruption has been associated with many negative health outcomes, such as cognitive impairment, metabolic syndrome, psychiatric illness. Night shift work is a very common form of circadian disruption. Between 15-20% of working population in the U.S. and Europe work in shiftwork positions [1].

### Circadian Disruption Impacts Health

Many researchers have reported that nightshift work is associated with many negative health outcomes, such as certain cancers, especially breast cancer [2, 3], aging and age-related diseases [4] and metabolic diseases [5]. But fewer studies have examined the effects of night shift work on maternal and child health. Although night shift work before and during pregnancy did not increase the risk of developing atopic dermatitis, asthma or hay fever of offspring [6], shiftwork during pregnancy has been associated with other health outcomes, including preterm delivery and miscarriage [7-9], babies born small for gestational age [7-9], preeclampsia and eclampsia [7, 8], gestational hypertension [7, 9], intrauterine growth retardation [8], gestational diabetes and meconium-stained amniotic fluid [9]. However, other studies have found that shiftwork during pregnancy was not associated with preterm birth [10]. Thus, the impacts of maternal shiftwork on offspring health are mixed, and the mechanisms that might link circadian disruption to offspring outcomes remain understudied.

### Epigenetic Mechanisms

The epigenome is one potential mechanism through which maternal shift work could impact offspring health. Epigenetics is studying the molecular features that can influence gene

expression potential without changing the DNA sequence, which includes DNA methylation and histone modification patterns. The process where methyl groups are added to the DNA is DNA methylation, primarily at cytosine bases. Instead of static, epigenetic changes are dynamic in different tissues and disease states, which indicates that epigenetic changes are potentially impacted by exposures and diseases and can be biomarkers to indicate exposures and diseases [11]. Many studies have shown that epigenetic changes are associated with prior environmental exposure, such as smoking during pregnancy, and that DNA methylation has potential as a biomarker to mark prior exposure [12]. Many animal and human longitudinal studies also indicate that early experiences play an important role in biological embedding that include DNA methylation [13]. Therefore, study of DNA methylation is vital for understanding child and maternal health. Night shift work has been associated with changes in placental DNA methylation, with implications for fetal health and development [14]. Placental DNA methylation studies are useful for identifying epigenetic variations shared by a group of subjects and associated with prenatal exposures that may impact the in-utero environment.

### Stochastic Epigenetic Mutations

Stochastic epigenetic mutations (SEMs) are an innovative approach for evaluating epigenetic disruption. The definition of SEMs is that, compared with the rest of population, an individual has an abnormal methylation value at a specific cytosine-phosphate-guanine (CpG) site. SEMs are rare and inconsistent between individuals and can occur throughout the genome, but extreme methylation levels of SEMs can associate with negative health outcomes, such as breast cancer, lung cancer, aging and age-related disease [15-17]. SEMs in placenta may reflect the placental health and may be associated with environmental exposures or prenatal stressors, but SEMs have not been thoroughly studied in placental tissue. It is important to test the effects of night shift work on variations of placental SEMs and to explore the related genes and pathways where SEMs occur in the placenta.

In this study, we investigated whether night shift work was associated with SEMs in placenta and conducted enrichment analysis to identify the genes and pathways where SEMs seem to occur in the placenta, which may have long-term health effects in the offspring.

## Methods

### Study Population

Our study population included 227 mother-infant pairs from the Rhode Island Child Health Study (RICHS). From 2009 to 2014, RICHS enrolled women between 18–40 years old and their infants at the Women and Infants Hospital of Rhode Island. Only full-term (37 weeks), singleton deliveries, without congenital or chromosomal abnormalities could be enrolled in this study. Informed consent based on the protocol approved by the Institutional Review Board of the Women's and Infants Hospital and Emory University had been all provided from all subjects [14]. Stochastic epigenetic mutations (SEMs) data was collected from the placenta, which were detected using two different measurements. The first measurement used the Illumina 450k arrays and the second used the Illumina EPIC chip. Placental sampling procedures, DNA methylation measurements, and processing of DNA methylation data are described in detail elsewhere [14]. SEMs are defined as that, for each CpG, an individual has a methylation value that is less than three times the inter-quartile range (IQR) below the first quartile, or more than three times the IQR above the third quartile [<Q1-(3*IQR), or >Q3+(3*IQR)]. This is consistent with the approach described by previous SEM research [15, 16]. Since we have methylation data from two separate arrays, we only capture these SEMs counts if a CpG is considered an SEMs from both of these two measurements. The total count of these SEMs across the entire microarray, for each placental sample is variable of focus for our analyses.

Questionnaires and clinical outcome information obtained from medical records was used to collect demographic information. We classified night shift work following the same approach as our prior work [14]. Interviewers obtain night shift work information by first asking “Have you ever worked outside the home?”, and if they say “Yes”, they would be asked to list all the jobs they have had, starting with their current or most recent job first. They were also asked to “Please indicate whether you worked a swing shift or a night shift on any of these jobs” [14]. For this analysis, we only include people who reported having a night shift as the most recent job.

In Table 1, demographic information of exposure, outcome and covariates are showed. “Job1 Non-shiftwork” and “Job1 Shiftwork” are women who did not have a nightshift job most recently and women who had a nightshift job most recently separately. “SEMs Counts” is the count number of SEMs. “Job1 Worked Hours” is weekly working time of participants. “Marital Status” indicates that participants are married, separated or single and “Maternal Education” indicates the education information of participants, which was leveled into two groups. “Maternal Ethnicity” shows their self-reported ethnicity, and they were grouped to white and non-white. “Maternal Gravita” means the total number of confirmed pregnancies a woman has had, which is also leveled into two groups. “Delivery Method” shows how did these mothers give birth, vaginal or cesarian section. “Birthweight (grams)” and “F13 Birthweight Group” both are information about the birthweight of infants and the former one is continuous and the latter one is categorical based on the Fenton growth charts [18]: SGA is small for Gestational Age, which means that infants are at or below the 10th percentile in birth weight and large for-gestational age (LGA) means infants are at or above the 90th percentile in birth weight, from an infant population of the same sex and gestational age, and Appropriate-for-Gestational-Age (AGA) means infant’s birth weight is between the 10th and 90th percentiles. “Gender” is the sex of the infants. “Household income” means the annual income of their family. “Maternal Smoking Status” indicates whether mothers reported any smoking during pregnancy.

A cumulative adversity score index was used to adjust for socioeconomic factors, which can avoid multicollinearity and increase degree of freedom. These factors include household income, household number, maternal education and marital status. The value of the adversity score was caculated by the following characteristics: if women whose median household income (adjusting for the number of people in the household) is below the federal poverty line for the year the infants were born, 1 point would be added; if women whose household was larger than 6, 1 point would be added, women who were single (never married) or separated (divorced), 1 point would be and women whose highest level of education was high school or less, 1 point would be added. Range of the cumulative adversity score is from 0 to 4, the increasing of score represents the level of adversity increases, which indicates the cumulative socioeconomic adversity of participants [1&8].

## Statistical Analyses

We explored differences in potential covariates between mother who did and didn't work night shifts via t-test (continuous) and $\chi^2$ tests (categorical). We then conducted linear regression by regressing placental SEMs on night shift work ("No"/ "Yes") without adjusting for any covariates. Next, we conducted linear regression adjusting for maternal age (years), maternal smoking status during pregnancy ("No"/ "Yes"), adversity score (0–4), and sex of the infant ("Female"/ "Male"), maternal ethnicity ("White"/ "Non-white"), maternal gravita ("1"/ "2+"), delivery method ("Vaginal"/ "Cesarian Section"), birthweight group ("AGA"/ "LGA" or "SGA"). We use 0.05 as alpha level and both p-values and 95% confidence interval were calculated for inference. As a secondary analysis, we also performed linear regression with winsorized SEM counts, with and without adjusting for covariates to reduce the impact of outliners of SEM counts.

We then conducted a Poisson regression modeling for SEMs and night shift work ("No"/ "Yes") without adjusting for any covariates, and also conducted Poisson model adjusting for same

covariates in the linear model with p-value and 95% confidence interval. To explore whether exclusion of smokers impacted our findings, we also performed a sensitivity analysis for both linear regression and Poisson regression. P-values and 95% confidence interval were calculated as well in sensitivity analysis (alpha is 0.05).

### Enrichment Analyses

To better understand whether placental SEMs were enriched in particular biological pathways or were randomly distributed throughout the genome, we performed enrichment analysis on the 29,693 CpGs that had placental SEMs and used 351,595 CpGs that overlapped between the 450K and EPIC arrays as background. We tested for enrichment with gene ontology (GO)-terms and KEGG pathways in R using the 'missMethyl' package [19]. The test for GO enrichment was not restricted to specific genomic locations. The test for KEGG enrichment was conducted without restriction first and conducted again with CpGs that were annotated to "TSS200" and "TSS1500" genomic locations, which represent genomic regions where DNA methylation is likely to impact nearby gene expression. Fisher's exact test ($p<0.05$) was used to calculate p-values for the enrichment analyses. One of the potential biases when we conduct enrichment analyses from DNA methylation studies is that some genes have more CpGs than others, and thus are more likely to show up by chance. 'missMethyl' package can perform gene set analysis in the context of DNA methylation array data for differential methylation of CpG sites and regions, while controlling for the different numbers of CpGs per gene [20].

## Result

### Study Population Demographics

Demographic information for the participants (n = 227) and covariates included for the epigenetic analysis are provided in Table 1. There are 48 women that reported night shift work and 161

women did not. Overall, women who reported working the night shift were more likely to be younger, single or never married, non-white, lower level of education, lower household income and their children have lower birthweight. While not statistically significant, women who worked the night shift trended towards a smaller number of confirmed pregnancies, smoking during pregnancy and higher adversity score.

### Characteristics of SEM Counts

SEM counts were highly variable in this cohort, with a range of 22 to 2358.The median (IQR) SEM counts is 366 (267.5 to 517.5). The distribution of SEM counts shows in Figure 1.

### Higher Number SEMs Associates with Night Shift Work

We conducted univariate linear regression analysis for all variables in Table 1 to assess which of these potential confounders were associated with placental SEM counts. We found that weekly worked time ($\beta = 0.32$; p=0.88; 95%CI = (-3.75, 4.40)), maternal age ($\beta = 0.32$; p=0.93; 95%CI = (-7.07, 7.72)) and birthweight ($\beta = -0.01$; p=0.83; 95%CI = (-0.06, 0.05)) were not associated with SEMs, so these three variables were not adjusted in the multiple linear regression and Poisson regression.

We then used linear regression to test whether the number of placental SEMs differed among mothers that had night shift work as their primary occupation. We found that maternal night shift working had a positive and significant association with SEM counts in unadjusted model ($\beta = 105.2$; p=0.01; 95%CI = (21.1, 189.3)). When maternal ethnicity, maternal gravita, delivery method, birthweight group, gender, maternal smoking status and adversity score were adjusted for, the association remained positive and significant ($\beta = 107.1$; p=0.02; 95%CI = (17.7, 196.6); Figure 1). We then performed the same analysis with winsorized SEM counts. The results of unadjusted linear model also show a positive association between maternal night shift working

and SEM count, but not statistically significant (β = 50.4; p=0.06; 95%CI = (-2.2, 102.9)). After adjusting for covariates, the winsorized linear regression results were also positive but again not significant (β = 45.0; p=0.10; 95%CI = (-8.8, 98.8)). Both of these winsorized results indicated that the beta coefficient gets much smaller, but the confidence interval is still indicative of a likely positive association.

The above models were performed with linear regression, so that our findings are comparable to what is reported in the published literature for the analyses of SEMs. We also used Poisson regression to test whether mothers that worked night shifts had greater counts of placental SEMs because the SEMs are count base data. Again, maternal night shift working had a positive and significant association with SEM counts in unadjusted models (β = 0.23; p=0.01; 95%CI = (0.05, 0.41)), and when adjusting for maternal ethnicity, maternal gravita, delivery method, birthweight group, gender, maternal smoking status and adversity score (β = 0.24; p=0.01; 95%CI = (0.05, 0.42); Figure 2).

### SEMs are Enriched in Certain Biological Pathways

To test whether SEMs were more likely to occur in genes that belong to certain biological pathways, and thus identify possible biological mechanisms that may be impacted by placental SEM formation, an enrichment test was conducted with biological pathways defined by GO and KEGG on the probes with SEMs. There were 351,595 CpGs at which an SEM could occur and 29,693 CpGs where SEMs were detected across both of our arrays.

For the GO test, of the 22,716 GO-terms tested, the CpGs with the most SEMs were significantly enriched in 94 of them (FDR<0.05). The top five GO terms results were “neuron projection morphogenesis”, “plasma membrane bounded cell projection morphogenesis”, “neuron

differentiation", "cell projection morphogenesis" and "neuron projection development". The top ten GO terms results are included in Table 2.

In Kyoto Encyclopedia of Genes and Genomes (KEGG) test, 338 pathways were tested. None of the KEGG pathways were significantly enriched. But interestingly, the top two significant pathways are "glycosphingolipid biosynthesis - globo and isoglobo series" and "nicotine addiction"; 13 out of 14 genes in the "glycosphingolipid biosynthesis – globo and isoglobo series" pathway had SEMs and 30 out of 40 genes in the "nicotine addiction" pathway had SEMs. We also ran a secondary analysis where the gene set enrichment analysis was restricted to CpGs annotated to the transcriptional start sites (TSS) "TSS200 and TSS1500" of different genes. TSS1500 refers to 200–1500 bases upstream of the TSS, while TSS200 refers to 0–200 bases upstream of the TSS; this region is also known as the promoter region and is highly sensitive to DNA methylation. "nicotine addiction" was the only pathway that was significantly enriched in this secondary enrichment analysis, where 22 out of 40 genes have SEMs in nicotine addiction pathway in these specific genomic locations.

## Sensitivity Analysis

A sensitivity analysis with maternal smoking status was conducted to test whether maternal smoking status plays a very important role in the relationship between maternal nightshift work and SEMs. After excluding maternal smokers, we reran the linear regression model and Poisson regression model adjusted for covariates. Excluding smokers, the coefficient is 95.99 in linear regression and 0.21 in Poisson regression, which were only slightly attenuated compared to our original findings.

## Discussion

While the association between nightshift work and DNA methylation levels shared by a population has been demonstrated before [14], we report the first analysis of the stochastic epigenetic mutations (SEMs) in placenta associated with maternal night shift working. Mothers with nightshift work tend to have more CpG sites with SEMs than those without nightshift work, even when controlling for maternal ethnicity, maternal gravita, delivery method, birthweight group, gender, maternal smoking status and adversity score. We also analyzed whether SEMs were enriched in any biological pathway or whether they were randomly distributed throughout the genome and found that placental SEMs are enriched in biological pathways involving cell morphogenesis, neuron differentiation, development and morphogenesis, glycosphingolipid biosynthesis and nicotine addiction.

Nightshift work is one of the most common occupational causes of circadian disruption, which has been found to have associations with several negative health effects. At present, there are multiple published studies on the influence of circadian disruption, especially nightshift work, on epigenetics. These are mostly focused on cancer and metabolic diseases. Javier et al., Reszka et al., Samulin Erdem et al. and Fagundo-River et al. found that breast cancer was associated with circadian disruption, especially night shift work, and that circadian gene methylation may be dysregulated in tumor tissue of breast cancer [21-23]. Cedernaes et al.'s study showed that epigenetics and transcriptional profiles of core circadian clock genes in metabolic tissues can contribute to disruption of metabolic integrity by nightshift work [24]. Kino et al. found that nightshift work can repress the mechanism of glucocorticoids to effect certain organ functions by epigenetic regulation [25]. While White et al. indicated that nightshift work is associated with epigenetic age acceleration, an epigenetic biomarker of biological aging [4]. All the studies show

negative health effects of night shifts, and potentially mediated by epigenetic variation, which we summarize in Table 3.

SEMs are an innovative approach for evaluating epigenetic disruption. Traditional epigenome-wide associations studies test for mean differences in methylation levels at each CpG site, while SEMs represent extreme methylation values at particular CpG sites relative to the rest of the study sample. SEMs have been associated with several negative health outcomes and some exposures which affect human health. Spada et al. found that the burden of SEMs in newborns was significantly higher in the preterm birth (PTB) group, which may associate with specific epigenetic signatures involving the immune system [26]. Gentilini et al. demonstrated that SEM counts were associated with hepatocellular carcinoma (HCC) tumors and hepatitis B and/or C virus infection status [27]. Gagliardi et al. also concluded that there is association between SEMs and different cancers [17]. Research from both Curtis et al. and Gentilini et al. showed that SEMs accumulate with age [15, 28]. Interestingly, Feinberg et al. used a different but related epigenetic metric, variably methylated regions (VMRs) that exhibit extreme variance in methylation levels, to demonstrate that VMRs contribute to development and morphogenesis in mouse and human liver, and in mouse brain [29]. These studies (Table 4) demonstrate that while research on SEMs is still limited in scope, studies have shown that SEM counts throughout the genome are related to many negative health outcomes, and it also could play an important role in the impact of certain environmental factors on human health.

While the impacts of night shift work on epigenetics and health have been studied, there are few studies that focus on the impacts during pregnancy and on reproductive tissues; only one study has focused on the placenta which serves critical functions during pregnancy. Clarkson-Townsend et al. compared DNA methylation of placental tissue of 53 nightshift working mothers and 184 non-nightshift working mothers from this same RICHS cohort that we studied for SEMs, and they identified significant associations between nightshift work and differential methylation

in the placenta [14]. Average absolute differences in DNA methylation for 298 CpG sites typically corresponding to roughly 1.7% changes in methylation at individual sites [14]. While our result showed that mothers with nightshift work had significantly greater numbers of SEMs, which represent extremely high or low DNA methylation levels at individual sites throughout the genome. However, SEMs occur at different locations throughout the genome for each individual, per their definition of being stochastic. Thus, our work builds upon this previous research, together demonstrating that nightshift work is associated with small differences in DNA methylation at specific CpG sites, and with an increased occurrence of stochastic extreme differences in methylation that are distributed throughout the genome.

Enrichment analyses were conducted to test whether CpGs with SEMs were enriched for certain biological pathways. GO-term enrichment analysis showed placental SEMs might be more likely to occur in genes involved in neuron development, differentiation and cell morphogenesis. This may indicate that areas of the placental genome involved in development and morphogenesis of cells and neurons are more likely to have SEMs. This is similar to the findings of Feinberg et al, in their study of variably methylated regions. They identified highly variably methylated regions, in liver and brain from human and mice samples, which tended to occur in genes involved in development and morphogenesis [29]. Interestingly, we also found that 13 out of 14 genes in the glycosphingolipid biosynthesis-globo series pathway had placental SEMs. Glycosphingolipid has been implicated in hyperlipidemia and glycosphingolipid synthesized in this pathway may accumulate in the artery wall and contribute to atherosclerosis [30], and thus is involved in metabolic activities. Additionally, the nicotine addiction pathway was significantly enriched for placental SEMs, and there is a strong relationship between DNA methylation and tobacco smoking. Thus, we conducted sensitivity analysis by excluding mothers that smoked during pregnancy to explore whether non-smoking mothers that worked the night shift still had greater SEM counts and found that our results were only slightly attenuated among non-smokers. We

have not tested whether placental SEM-enriched pathways differ between nightshift and non-night shift work group, or within specific individuals. Future studies could compare whether there are similar, or different SEM-enriched biological pathways, based on prior exposures including but not limited to night shift work

A number of limitations need to be considered when interpreting our results. First, although the number of subjects in our study is similar to previous study of EWAS with DNA methylation, a larger sample size could allow for exploration of stratified analyses by different job types. Second, previous studies found that the time of night shifts significantly associated with DNA methylation, but we only collected weekly working hours information and found that weekly working time do not have significant association with SEMs. Future studies could collect monthly, annual and total time of nightshift working information and include them in analysis. However, this study does have several strengths. We use two different methylation arrays to identify the SEM counts in the placenta, and only included those that were replicated across these two arrays in our analysis; this reduces the chances that the extreme methylation values captured by SEMs were influenced by measurement error. Additionally, we studied SEMs in the placenta, which has implications for maternal and child health and also can reflect the placental health and may be associated with environmental exposures or prenatal stressors.

## Conclusions and Future Directions

In conclusion, this study builds on previous research that observed shiftwork associated differential methylation [14], by demonstrating that shift work is also associated with greater numbers of placental SEMs. Additionally, since this is the first study of SEMs in human placenta, that we are aware of, this demonstrates that SEMs offer an innovative alternative approach to studying the impacts of environmental exposure on placental epigenetics. We also characterize the biological pathways in which placental SEMs tended to occur, including neuron development,

differentiation and morphogenesis, glycosphingolipid biosynthesis and nicotine addiction, which may give us insights of how SEMs are formed, and which biological pathways in the placenta are more likely to be impacted by SEMs. These studies about SEM are important for understanding the variety of negative health outcomes induced by nightshift work. More studies are warranted to examine associations between prenatal exposures, reproductive and birth outcomes, and the stochastic epigenetic mutations in the placental epigenome. Because our study focused on placenta tissue, future studies to examine whether SEMs in other tissues are associated with night shift could be considered. Additionally, to incorporate transcriptome and metabolome data is another direction, which could help us understand whether these SEMs impact the biological functions of the placenta. This study did not include the association of health outcomes, so others could also examine whether placental SEMs are associated with health outcomes in children, which may be an early indicator in utero stress that impacts fetal development.

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| | Non-nightshift Work (n= 161) | | NightShift Work (n= 48) | | p-value |
|---|---|---|---|---|---|
| **Variable Name** | **Mean/N** | **SD/%** | **Mean/N** | **SD/%** | |
| **SEM Counts** | 402.9 | 215.3 | 508.1 | 372.2 | 0.07 |
| **Worked Hours (NA=16)** | 37.4 | 7.2 | 39.92 | 13.1 | 0.21 |
| **Maternal Age (years)** | 31.52 | 4.5 | 29.33 | 5.1 | <0.05 |
| **Marital Status** | | | | | <0.05 |
| Married | 129 | 80.1% | 27 | 56.3% | |
| Separated or divorced | 5 | 3.1% | 2 | 4.2% | |
| Single, never married | 27 | 16.8% | 19 | 39.6% | |
| **Maternal Education** | | | | | <0.05 |
| College graduate or more | 107 | 66.5% | 21 | 43.8% | |
| High school or less | 54 | 33.5% | 27 | 56.3% | |
| **Maternal Ethnicity** | | | | | <0.05 |
| White | 133 | 82.6% | 32 | 66.7% | |
| Non-White | 28 | 17.4% | 16 | 33.3% | |
| **Maternal Gravita** | | | | | 0.15 |
| 1 | 35 | 21.7% | 16 | 33.3% | |
| 2+ | 126 | 78.3% | 32 | 66.7% | |
| **Delivery Method** | | | | | 0.98 |
| Cesarian Section | 78 | 48.4% | 24 | 50.0% | |
| Vaginal | 83 | 51.6% | 24 | 50.0% | |
| **Birthweight (grams)** | 3598 | 640.1 | 3345 | 693.6 | <0.05 |
| **Birthweight Group** | | | | | 0.12 |
| AGA | 99 | 61.5% | 24 | 50.0% | |
| LGA | 41 | 25.5% | 12 | 25.0% | |
| SGA | 21 | 13.0% | 12 | 25.0% | |
| **Gender** | | | | | 1 |
| Female | 82 | 50.9% | 24 | 50.0% | |
| Male | 79 | 49.1% | 24 | 50.0% | |
| **Household Income (NA=1)** | | | | | <0.05 |
| $100,000 or more | 56 | 35.0% | 7 | 14.6% | |
| $50,000-$99,999 | 61 | 38.1% | 11 | 22.9% | |
| under $49,999 | 36 | 22.4% | 29 | 60.4% | |
| Not Sure or Refusal | 7 | 4.4% | 1 | 2.1% | |
| **Maternal smoking during pregnancy (NA=1)** | | | | | 0.48 |
| YES | 7 | 4.4% | 4 | 8.3% | |
| NO | 153 | 95.6% | 44 | 91.7% | |
| **Adversity Score (NA=10)** | | | | | <0.05 |
| 0 | 89 | 55.3% | 17 | 35.4% | |
| 1 | 37 | 23.0% | 12 | 25.0% | |
| 2 | 21 | 13.0% | 12 | 25.0% | |
| 3 | 5 | 3.1% | 5 | 10.4% | |
| 4 | 0 | 0.0% | 1 | 2.1% | |

Table 1. Demographic information of participants (n = 227) by night shift work status. AGA is short for appropriate-for-gestational-age, which is defined that if the birthweight is between the 10th and 90th percentiles for the infant's gestational age and sex, the birth would be considered to be appropriate for gestational age. SGA is short for small for gestational age and LGA is short for large for gestational age. SGA and LGA respectively mean that, from an infant population of the same sex and gestational age, infants who are at or below the 10th percentile in birth weight or are at or above the 90th percentile in birth weight. P-values are generated between non-night shift and night shift workers by using $\chi 2$ test, Fisher's exact test for categorical variables and 2-sided t-test for continuous variables between non-night shift and night shift workers.

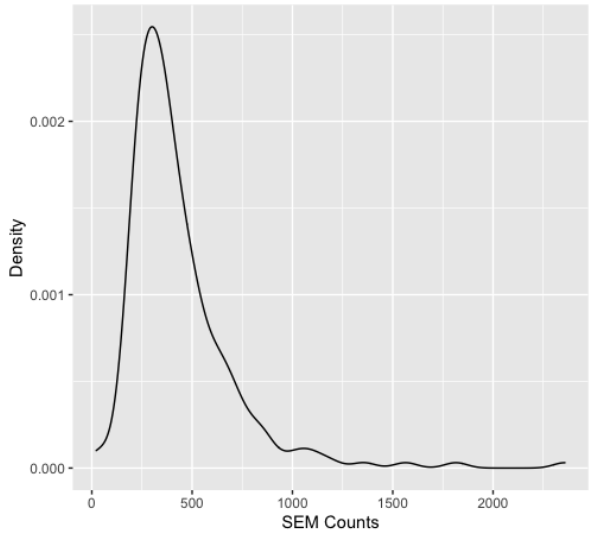


Fig 1. The distribution of SEM counts.

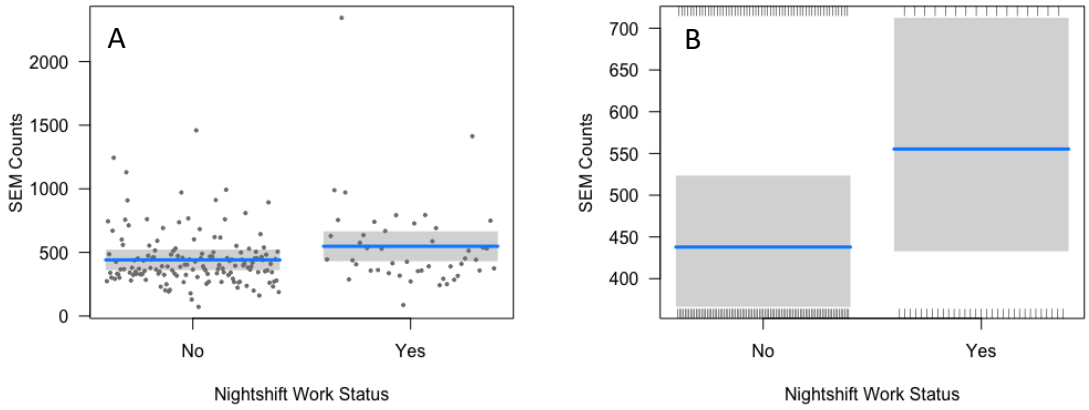


Fig 2. Results of placental SEMs and night shift work. A. Partial box plot from linear regression results, adjusted for maternal ethnicity, maternal gravita, delivery method, birthweight group, gender, maternal smoking status during pregnancy and adversity score. The dark gray dots mean individual's respectively SEM counts in two groups. The blue horizontal lines show expected value of SEM counts of two different work status groups. The gray bands are confidence intervals for the expected value of two groups' SEM counts. B. Partial box plot of Poisson regression results, adjusted for maternal ethnicity, maternal gravita, delivery method, birthweight group, gender, maternal smoking status during pregnancy and adversity score. The blue horizontal lines show expected value of SEM counts of two different nightshift work status respectively and the gray bands are confidence intervals for the expected value of two groups' SEM counts.

| | ONTOLOGY | TERM | N | DE | P.DE | FDR |
|---|---|---|---|---|---|---|
| **GO:0048812** | BP | neuron projection morphogenesis | 662 | 451 | 3.57E-11 | 8.11E-07 |
| **GO:0120039** | BP | plasma membrane bounded cell projection morphogenesis | 676 | 457 | 1.27E-10 | 1.42E-06 |
| **GO:0030182** | BP | neuron differentiation | 1389 | 867 | 1.87E-10 | 1.42E-06 |
| **GO:0048858** | BP | cell projection morphogenesis | 680 | 457 | 3.55E-10 | 2.02E-06 |
| **GO:0031175** | BP | neuron projection development | 996 | 635 | 6.40E-10 | 2.91E-06 |
| **GO:0048667** | BP | cell morphogenesis involved in neuron differentiation | 599 | 407 | 9.58E-10 | 3.63E-06 |
| **GO:0000904** | BP | cell morphogenesis involved in differentiation | 747 | 493 | 1.15E-09 | 3.72E-06 |
| **GO:0032990** | BP | cell part morphogenesis | 700 | 465 | 1.48E-09 | 4.20E-06 |
| **GO:0000902** | BP | cell morphogenesis | 1032 | 651 | 4.89E-09 | 1.14E-05 |
| **GO:0048666** | BP | neuron development | 1129 | 706 | 5.03E-09 | 1.14E-05 |

Table 2. The 10 GO terms with the smallest p-values in the 22716 GO test. The first column is the number of GO terms. ONTOLOGY shows the GO term belongs to biological processes (BP). TERM presents each different GO term. N is the number of total genes in the GO term. DE is the number of genes within that GO term that had SEM CpGs. P.ED is the p-value for over-representation of the GO term in the set. FDR is the false of discovery rate.

| Study | Gene IDs | Health Outcomes |
|---|---|---|
| Reszka et al, 2017 | *BMAL1, BMAL2, CLOCK, NPAS2, CRY1, CRY2, PER1, PER3, TIMELESS* | Breast cancer |
| Samulin Erdem et al, 2017 | *CLOCK*  *BMAL1*  *CRY1, PER1* | Breast Cancer |
| Fagundo-Rivera et al, 2020 | | Breast Cancer |
| Cedernaes et al, 2015 | *CLOCK* | Metabolic Integrity Disruption |
| Kino et al, 2011 | *CLOCK* | Glucocorticoid |
| White et al, 2019 | *ZFHX3* | Age Acceleration |

Table 3. Summary of papers related to nightshift work and epigenetic variation.

| Study | Potential Association |
|---|---|
| Spada et al, 2020 | PTB exposure |
| Feinberg et al, 2009 | Development and morphogenesis in mouse and human liver and mouse brain |
| Gentilini et al, 2017 | HCC, hepatitis B and/or C virus infection status |
| Gagliardi et al, 2020 | Higher risk of different cancers in prediagnostic blood samples |
| Curtis et al, 2019 | PBB exposure, age |
| Gentilini et al, 2015 | The correlation between XCI skewing and aging |

Table 4. Summary of papers related to SEMs. (PTB = preterm birth; HCC = hepatocellular carcinoma, PBB = polybrominated biphenyl, XCI= X chromosome inactivation)